A STUDY OF THE TEMPORAL DISTRIBUTION OF BROMSULPHTHALEIN AND ITS ROLE IN THE ASSESSMENT OF LIVER CELL DAMAGE IN DIFFERENT ANIMAL SPECIES

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I N D E X

XI. RESULTS (CONTD.)

I. GENERAL INTRODUCTION.

It has been noted since the latter part of the nineteenth century that certain dyes, when administered to the body are removed from the circulation and eliminated almost entirely by the liver into the bile. This is similar to the dye phenolsulphonphthalein (phenol red) which is almost entirely excreted by the kidneys into the urine. It was originally considered that the elimination of dyes by the liver was the primary function of the Kupffer cells, part of the reticulo-endothelial system. This is true for certain dyes such as trypan blue, but fluorescence microscopy has shown that rose bengal, uranin (Fluorescein) and azorubin-S are taken up by the hepatic cells. It was assumed that sodium phenoltetrabromphthalein sulphonate, usually known as bromsulphthalein, or BSP would be treated in the same manner as rose bengal, and this was confirmed later by using S^{35} labelled bromsulphthalein.

Since the introduction of the halogenated phthaleins into experimental and clinical use for investigating liver function, a variety of methods have been proposed for estimating the ability of the liver to eliminate these substances. Originally the quantity

of dye excreted in the faeces was estimated, but the obvious technical difficulties inherent in this procedure made the results of the test very doubtful. With the introduction of duodenal intubation, attempts were made to assess the state of liver function by determining the time of appearance and disappearance of dye in the bile and the total quantity of dye eliminated during varying periods of study. Accurate quantitative estimations of the dye, however, were difficult due to the relative crudity of the methods used. These methods have now largely been abandoned. Nowadays, the common practice is to determine the rate of removal of the dye from the blood stream, and the disappearance rate is assumed to be an indication of hepatic function. The dye most commonly used is bromsulphthalein, but until recently there has been little knowledge of the mechanism of its removal from the body.

There is no best test of liver function even for one purpose. The bromsulphthalein liver function test being the one most commonly used in clinical practice to assess the ability of the liver to deal with an exogenous dye. Since the liver performs a multitude of functions several tests are required to obtain the maximum of information and indeed detect impaired function. It is therefore important that

the rationale and mechanism of the individual tests should be understood.

The object of this thesis has been to study the normal pattern of liver function as indicated by the dye bromsulphthalein in different animal species but especially the dog as well as human volunteers. The content of dye in various body fluids by colorimetric methods has been investigated and regression formula derived for its estimation in these fluids. The temporal distribution of bromsulphthalein has also been studied and a mathematical model is given which suggests that a modified bromsulphthalein liver function test will yield more information than is given by the standard clinical test. In an attempt to simulate various degrees of liver damage substances which are known to enter the liver cells have been given and their effects on bromsulphthalein excretion observed and analysed. It is hoped that the value of bromsulphthalein as a tool in the study of liver physiology will become apparent from this work and it is suggested that the adoption of the modified test may prove of use clinically.

A detailed description is given in the Introduction of the development, structure and function

of the liver initially followed by a review of the literature on the ability of the liver to deal with exogenous dyes and bromsulphthalein in particular. This rather comprehensive survey is considered essential to any assessment of the value of bromsulphthalein as a tool in the study of liver physiology, since the liver is a complex organ and cannot be adequately described briefly.

II. INTRODUCTION.

The Liver.

A consideration of certain aspects of its development, anatomy and functions as well as a brief outline of liver function tests.

1. GROSS ANATOMY.

The necessary information for this and the following sections has been derived from Maximow and Bloom, 1948, Samson Wright, 1952, Johnston and Whillis, 1954, Cantarow and Trumper, 1955, Evans, 1956, Popper and Schaffner, 1957, unless stated otherwise.

The liver is the largest gland in the body and it lies immediately below the dome of the diaphragm, occupying the upper portion of the abdominal cavity, mainly to the right of the midline. It is a pyramidal shaped organ with its apex towards the left and its base to the right, the sides forming anterior, posterior, superior and inferior surfaces. It is to a large extent under cover of the lower ribs and costal cartilages on the right side, the xiphoid process and some of the costal cartilages on the left side. It secretes a yellowish-green or brown fluid, of bitter taste called bile. The bile is conveyed from it by two (or more) hepatic ducts which unite to form a common hepatic duct. In those species with a gall bladder, the common hepatic duct is joined by a duct (the cystic duct) from the gall bladder to form the common bile duct which conveys the bile to

the duodenum. The gall bladder, a pear-shaped sac, is attached to the liver and is connected to the bile duct by the cystic duct, through which bile can pass to and from the gall bladder. The gall bladder acts as a reserve store of bile which the liver is continuously secreting. It also concentrates the bile, probably by the absorption of water (Ivy, 1934). In those species without a gall bladder, the hepatic ducts may unite to form a common duct which empties into the duodenum or they may open into the duodenum separately. The weight of the liver is approximately one fortieth to one fiftieth of the body weight in the adult of most species. The size is to some extent dependent upon the metabolic importance of the liver. The liver is relatively much larger in smaller animals, in the newborn and in the young because of the higher metabolic rate. The liver is also relatively much larger in carnivores than in herbivores. In omnivores, such as man, it has an intermediate size. Elias, 1952, pointed out that although there are remarkable variations in the embryonic development of the liver in different species, the end product is similar in all the more highly developed animals.

The liver is reddish brown in colour, and as

it is almost entirely covered with peritoneum, the surfaces are smooth and glistening. Within this serous coat is a connective tissue capsule (Glisson's capsule). At the hilum of the liver (the transverse fissure or porta hepatis) where the blood vessels enter the gland, the capsule surrounds the vessels and enters with them so that it surrounds and supports even the smallest branches of the vessels. The liver is firm but friable to the touch and is readily lacerated. The lacerated or cut surface of the liver is granular and a pattern of red dots is interrupted by grey areas representing portal tracts. The distance between these red dots varies from half to two millimetres, and this is the diameter of the hepatic lobule. Each lobule is polygonal or irregular in form, surrounded by connective tissue in which the vessels, nerves and bile capillaries lie. The cells comprising the parenchyma of the liver are thus divided up into lobules. In most mammals the boundary of these lobules is not easy to see, with the notable exception of the pig, where each lobule is outlined by a thick layer of connective tissue.

The liver is a very vascular organ and receives its blood supply from two sources. Arterial (oxygenated)

blood is supplied by the hepatic artery for the substance of the liver. Venous blood is carried to the liver by the portal vein; this blood contains materials absorbed from the alimentary canal upon which the liver cells exert their actions. Both these vessels break up into right and left branches in the porta hepatis. These vessels break up within the liver into small branches and run along together with the tributaries of the bile ducts at the periphery of the liver cells in the sinusoids, which are in contact with the cells, to the centre of each lobule. Here, the mixed hepatic and portal blood is received into a central vein. The central veins from all the lobules unite together to form hepatic veins. The hepatic veins open directly into the inferior vena cava which lies in a groove at the back of the liver.

2. THE FUNCTIONS OF THE LIVER.

The liver performs a multitude of diverse functions and it is only possible to give an outline of some of these functions in this section. The liver is situated within the cardio-vascular system so that all the blood coming from the gastro-intestinal tract has to pass through the portal vein before reaching the heart. This blood filters through the liver via the

sinusoids, which contain cells of the reticulo-endothelial system (Kuppfer cells) in their walls (Aschoff, 1924). The Kuppfer cells prevent any particulate material from reaching the main circulation (Doan, 1940; Popper, Gyorgy and Goldblatt, 1944; Knisely, Bloch and Warner, 1945). Because the sinusoids containing the blood are in contact with the liver cells, the latter are able to absorb, store and metabolise the nutrient material from the gastro-intestinal tract.

The liver cells perform storage, anabolic, catabolic, secretory (exocrine and endocrine) and excretory functions. In the liver, more than in any other organ, the metabolism of carbohydrate, fat and protein is interwoven so that smaller metabolites of these substances are common breakdown as well as common building stores of carbohydrate, fat and protein. These small metabolites constitute a common metabolic pool. The metabolic pool in the liver serves not only the needs of the liver, but also the whole body. Carbohydrates, fat and protein are potential precursors of each other by means of common pathways through the metabolic pool.

The energy necessary for the metabolic transformations in, to and from the metabolic pool is

provided by the breakdown of high-energy phosphate bonds (about 10-12,000 calories of energy per bond). The nucleotide, adenosine triphosphate (ATP) by losing a high-energy phosphate bond, becomes adenosine diphosphate (ADP) with the rapid release of energy. There are two main sources which provide the necessary energy and also restore the high-energy bonds to reform ATP from ADP. The most efficient source is the aerobic oxidation of substances in the metabolic pool to carbon dioxide and water via the tricarboxylic acid cycle of Krebs (1943). The other source is from glycosis (a stage in the breakdown of glycogen to pyruvic acid). The former process provides about thirteen times as much energy as the latter.

The liver plays an important part in the metabolism of almost all vitamins, storing some and altering others. The liver stores about 95% of the total vitamin A content of the body (Moore, 1931), and is also the main site for storage of vitamin D. Most of the known vitamins of the B complex are constituents of respiratory enzymes and are therefore related to liver function. Most of them are specifically connected with the liver and the majority of them are stored there. Vitamin K is necessary for

the formation of the glycoprotein prothrombin, which is essential for blood coagulation.

The bile acids are a specific metabolic product of the liver. Chemically, the bile acids are related to a group of substances with a similar ring structure (the cyclopentanophenanthrene nucleus) such as cholesterol, steroid hormones, digitalis, vitamin D and some carcinogenic hydrocarbons. In alkaline solutions such as bile, the bile acids are sodium salts. There are many natural, as well as synthetic bile acids known and great variations are present in different species and these have been reviewed by Berman, Snapp, Ivy, Atkinson and Hough, 1941, Haslewood and Wooten, 1950 among others. The evidence that bile acids are formed by the liver is based on observations of the blood and bile concentrations of bile acids after hepatectomy or liver damage. In the hepatectomised dog the blood and urine are free of bile acids, but any injected bile acids are recovered in the urine (Bollman and Mann, 1936). The bile acids excreted in the bile are largely re-absorbed in the intestine and taken up by the liver and then eliminated again in the bile by the liver (i.e. the enterohepatic circulation of bile salts). The greater part of the bile salts in the bile are thus eliminated

from the blood and a small part are synthesized in the liver cells.(Josephson, 1941). The bile salts have a stimulating effect on the biliary flow as well as other properties.

The liver is involved in protein metabolism, both in the formation and breakdown of individual proteins. It provides the simple precursors from the metabolic pool in order that tissue-protein can be formed, as well as the formation of its own protein. It is the principle site for the synthesis of serum proteins. The liver synthesises all the plasma albumin and fibrinogen and much of the globulin is formed outside the liver (Wiggers, Opdyke and Johnson, 1946). It is also a site for storage of protein. The liver, as well as other tissues, breaks down protein into amino-acids and polypeptides. Together with the kidney and brain, the liver deaminates amino-acids. It is the only organ, in mammals, which forms urea from the nitrogen obtained by deamination. It may also discharge amino-acids into the blood. Some transformations of amino-acids are preferentially located in the liver, and some phases of the breakdown of protein complexes, such as nucleoproteins, with uric acid formation occur almost entirely in the liver.

The liver plays a predominant role in carbo-

hydrate metabolism of the body. Glucose is the main supply of energy for the body and it is transported in the blood to the tissue cells. In the fasting state, all the blood glucose originates from the liver. In liver damage, the importance of this is reflected in the sacrifice of almost all other functions of the liver for the production of blood sugar. The liver is the first organ to receive the monosaccharides from the gastro-intestinal tract and it converts all hexoses to the polysaccharide glycogen. The liver also metabolises other carbohydrates such as pentoses, and it has the ability to convert some of the metabolites of protein and fat to glycogen. Various breakdown products of glycogen and lactic acid are formed in many tissues of the body, especially muscle, and enter the blood stream to reach the liver where they are converted to glycogen. Hepatic glycogen and blood sugar are in equilibrium (Soskin and Levine, 1946). The reversible process of glycogen $\rightleftharpoons$ glucose formations represents part of a cycle which makes glucose available primarily for maintaining the blood glucose level. Glycogenolysis is regulated by the level of blood glucose and by hormones. Adrenal cortical extract (Seckel, 1940) and insulin (Cohn, 1946;

Soskin and Levine, 1946) have an inhibitory effect. Stimulation of the thyroid or adrenal medulla and splanchnic nerves, as well as acidosis and anaemia, promotes glycogenolysis (Peters and Van Slyke, 1946). The pancreatic hyperglycaemic factor, glucagon, increases hepatic glycogenesis by its effect in activating certain phosphorylating enzymes (Kibler and Myers, 1953). Thus, the role of the liver in carbohydrate metabolism consists in the formation of glycogen from monosaccharides and other metabolites, and the breakdown of glycogen to glucose with its controlled discharge into the blood-stream, as well as the metabolic conversion of sugar to protein and fat and vice versa.

The liver stores fatty acids as neutral fat, as phospholipids and to lesser extent cholesterol esters as well as being intimately concerned in the metabolism of neutral fat, phospholipids and cholesterol. The quantity and type of fat present in the liver varies in each species. This is the result of new formation, destruction and deposition of fat absorbed from the gastro-intestinal tract or mobilised from various parts of the body. The liver controls the absorption of fats from the gastro-intestinal tract by its secretion of bile acids into

the bile. It synthesises various lipids, forms serum lipids and aids in the distribution of lipids throughout the body. The liver is the main site of formation of its own and plasma-phospholipids as well as their destruction. The phospholipids are integral constituents of all cells and play a part in the oxidation of fatty acids (Di Luzio and Zilversmit, 1952) and in the stabilization of serum colloids (Ahrens, and Kunkel, 1949). The liver is the sole source of ketone bodies which are formed from fatty acids and may serve as a fuel for some body tissues. Cholesterol occurs in the body in the free alcohol state as well as a fatty acid ester. In most of the cells of the body, cholesterol exists in the free alcohol form, the ester form only being found in the blood, liver and intestine. Synthesis of cholesterol in the liver has been demonstrated (Srere, Chaikoff, Treitman and Burstein, 1950, Taylor and Gourley, 1950) and it apparently depends on the nutritional state (Tomkins and Chaikoff, 1952, Frederickson, Loud, Hinkelman, Schneider and Frantz, 1954). The liver is also the chief site of degradation of cholesterol (Hotta and Chaikoff, 1955).

The liver acts as a storehouse of water.

It influences the fluid balance of the body, due partly to sphincters in the hepatic vasculature and partly due to specific functions of the hepatic parenchyma. Water is poorly excreted in liver disease, depending upon the type and severity of the disease (Aldersberg and Fox, 1943). Aldersberg and Fox also found abnormal water tolerance tests in dogs intoxicated with phosphorus or histamine. During the acute stages of hepatitis, oliguria is a common event and diuresis heralds recovery (Jones, 1936). In acute hepatitis, the plasma volume is increased and plasma and urinary chlorides are decreased (Labby and Hoagland, 1947). In cirrhosis of the liver, low plasma volumes are frequent (Hyde, Berline, Parsons, Lawrence and Port, 1952), although increases in plasma volume have been reported (Perera, 1946). In some liver diseases, disturbed water metabolism is related to ascites formation.

The liver destroys oestrogen hormones and the pituitary antidiuretic hormone. It is probable that hepatocellular dysfunction may either alter the formation of substances influencing water metabolism (Baez, Mazur and Shorr, 1950; Shorr, Baez, Zweifach, Payne and Mazur, 1950; Lichtman, 1953) or the reduced

destruction of antidiuretic substances formed outside the liver (Pick, 1929).

In man and most animals, bile is continuously secreted by the liver cells (Whipple, 1922). Its expulsion from the gall bladder (if present) and its passage along the common bile duct into the duodenum is intermittent, related in time to the arrival of food in the intestine and is completely independent of the actual secretion by the liver. The bile is a highly complex fluid and its constituents differ somewhat in individual species. The physiological significance of many of its constituents is unknown, indeed some are only present in minute quantities and are almost certainly waste products undergoing elimination. The principal organic constituents (making up over 60% of the total biliary solids) are bile salts, bile pigments, cholesterol and lecithin. The bile salts and cholesterol have already been discussed. Lecithin is usually found together with cholesterol in most of the cells of the body and a small quantity is always present in the bile. The bile pigments are bilirubin and biliverdin, the latter being an oxidative derivative of bilirubin. Bilirubin is derived from haemoglobin, being the porphyrin (globin-free and iron-free) fraction of the haemoglobin molecule.

It is now accepted that the elements of the reticulo-endothelial system situated in various parts of the body, spleen, lymph-glands, bone marrow and the general connective tissues are responsible for the formation of the bile pigment. Of these, the bone marrow is probably the most important (Mann, Sheard and Bollman, 1926). It has been shown by Rich (1924) that the haemoglobin-bilirubin transformation effected by the reticulo-endothelial cells is an intracellular process. The bilirubin formed circulates in the blood stream bound to protein and is excreted by the liver cells from the vascular capillaries into the bile channels as the sodium salt (Watson, 1946). There is no evidence that the true secretory cells of the liver play any part in the formation of bilirubin; they merely excrete the pigment that reaches them preformed. The bile pigments serve no digestive purpose, and are partly excreted in the urine and the faeces. In the bile passages biliverdin is formed as an oxidation product of bilirubin. In the intestine, the bile pigments are reduced to urobilinogen. Urobilinogen is excreted in the faeces and on exposure to air is oxidised to urobilin. Some of the urobilinogen is reabsorbed from the intestine into

the portal blood and reaches the liver where it is largely re-excreted into the bile. A little of the urobilinogen, however, reaches the systemic circulation to be excreted in the urine as urobilinogen (which on exposure to air becomes urobilin).

By the chemical processes of oxidation, conjugation, reduction, methylation and acetylation, the liver detoxifies most of the digestive end-products. It is by these processes that harmful endogenous and exogenous substances are rendered harmless, as well as the pathways for non-toxic substances. The end-products are not always less toxic than the original substances but they may be more soluble and readily excreted in the urine. Many pathways for these transformations exist and there are marked variations in different species (Williams, 1947; Bray, Thorpe and White, 1951). Examples of some of these processes are the inactivation of many of the steroid hormones (oestradiol, progesterone, and testerone) by oxidation, the conjugation of glycine with benzoic acid, salicylic acid and para-aminosalicylic acid, glucuronic acid with camphor, menthol, phenol, benzene or morphine, acetylation occurring primarily with sulphonamides and paramino-benzoic acid and the coupling of indol with sulphuric acid to give indoxyl sulphate. The liver also excretes into the bile heavy

metals and certain drugs.

In the embryo and the first week after birth the liver serves to make red blood cells, but later it ceases to do this. It stores and possibly manufactures the antipernicious anaemia factor (Erythrocyte maturing factor) and it also stores iron and copper. The muscular tissues (particularly of the limbs) and liver wherein numerous chemical reactions are carried out, are the main sources of the body's heat. The liver thus plays a role in the heat regulation of the body.

Complex as the structure of the liver is, this remarkable diversity of function cannot be correlated with the structural elements. In general, all we can say is that the blood supply of the liver is arranged so that its cells are bathed in a plentiful supply of blood.

3. LIVER FUNCTION TESTS.

The problem of investigating liver function has proved to be difficult owing to the multitude of activities performed by this organ, and also to the diverse nature of the functions which must be evaluated. The activities of the liver (as mentioned in the previous

section) include the secretion of bile; the production of glycogen from glucose and other monosaccharides and from non-carbohydrate sources; the conversion to urea of ammonia liberated in the breakdown of amino-acids; the formation of some of the plasma proteins; the synthesis of a variety of substances needed by the body for its metabolic processes; the formation, from substances absorbed into or produced by the body, of products generally of lower toxicity and greater solubility suitable for excretion in the urine and indeed many other functions. The various activities of the liver cannot be arranged in order of importance to the body as a whole, nor does the efficiency of each activity decrease in any fixed order if the liver is damaged or its function interfered with in any way.

Many liver tests have been proposed, some of which are still performed as originally described, others having been modified or improved upon, and others having been replaced or given up. There is no <u>best</u> test of liver function even for any one purpose. Different species or individual members of the same species respond differently to the same test. For this reason , and because the reserve of the liver is so great, the best results can only be achieved by using

several tests. Although this research is only concerned with one particular test and its significance, a brief outline of those tests which are generally considered to be most helpful in human subjects is given (Stewart and Dunlop, 1949; Cantarow and Trumper, 1955; Popper and Schaffner, 1957).

For the purposes of assessing liver function, one is limited to those activities which are susceptible of quantitative measurement and are peculiar to the liver, or very nearly so, in order that the effect of other tissues does not mask changes in the amount of work done by the liver. The tests must also be sensitive enough quantitatively or qualitatively to detect any changes before the amount of impairment of liver function becomes extreme. Certain important functions cannot be assessed, for example, the part played by the liver in the formation of haemoglobin and the red blood corpuscles. Following the injection or absorption of glucose about ninety per cent of it will be converted to glycogen in the liver, and this process can be followed by estimating the blood glucose levels. This would not, however, give a true estimate of liver function because the conversion of glucose to glycogen also occurs in the muscles and it is considerably

effected by the production of insulin. Glycogen is also formed from fructose and galactose, a process which takes place in the liver and is not affected by insulin; these sugars can be estimated quantitatively and do provide the basis for tests of liver function. Clinically, liver function tests are required for the detection of functional impairment, prognosis, i.e. to assess the improvement during treatment, and diagnosis. These three purposes are not completely served by the same tests, though some are overlapping.

Liver function tests can be grouped in many ways. They are grouped here according to the aspect of liver function they are designed to test:-

(a) <u>Estimation of the capacity of the liver to metabolise a test dose of substances such as laevulose or galactose.</u>

These substances are metabolised only after synthesis to glycogen. Precise information is only obtained if quantitative estimations are made of the blood level of the sugar administered. Estimation of the total blood sugar is useless for this purpose. The test is carried out by a method similar to that used in applying the glucose tolerance test in diabetes. The laevulose

test consists of giving 50 gm. of laevulose (orally) after a sample of blood has been obtained and four further samples are withdrawn at half-hourly intervals. Normally the fructose concentration never rises above 20 mg./100 ml. (usually half to one hours after the test) and falls to about 5 mg./100 ml. after two hours. The galactose tolerance test is carried out in a similar manner. The concentration of galactose should normally never exceed 40 mg./100 ml. (Althausen, Lockhart and Solly, 1940; Meranze, Likoff and Schneeberg, 1942), blood samples being taken at 30 and 60 minutes. In these respective tests values greater than those given are indicative of hepatic insufficiency.

(b) Estimation of the blood level of substances excreted by the liver.

Either products of normal metabolism, such as bilirubin, phosphatase and cholesterol or foreign substances introduced intravenously may be used. Of the latter, the dye bromsulphthalein, a constant proportion of which is normally removed by the liver per unit time, is frequently employed.

Serum bilirubin concentration depends upon the rate of removal of bilirubin formed from the destruction of haemoglobin. Increased bilirubin concentrations in the blood may result from (i) increased destruction of haemoglobin or (ii) decreased excretion or retention, due to either cellular or excretory duct disease of the liver. Although 'direct' and indirect types of bilirubin are determined conventionally to differentiate the free bilirubin (direct) from the bound variety (bilirubin globin), the most recent chemical investigations reveal that there is actually only one form of bilirubin, the protein bound. Other substances may be present in the sera that produce alteration of the chemical reaction so as to yield varying levels of the 'two forms' of bilirubin. The maximal normal values for the direct and indirect forms are respectively 0.25 and 1,0 mg./100 ml. of plasma (Zieve, Hill, Hanson, Falcone and Watson, 1951).

The liver normally contains the enzyme alkaline phosphatase and excretes it in the bile, so that elevation of the serum alkaline phosphatase may be a manifestation of retention. At least this is

the convenient explanation for the observation that serum alkaline phosphatase increases in obstructive jaundice (Tallroth, 1949; Welin, 1952).

With disease or damage of liver parenchyma serum cholesterol and cholesterol esters may diminish. With biliary obstruction the total cholesterol may rise, but the cholesterol esters concentration or the ratio of ester to total cholesterol may diminish. If the cholesterol ester concentration is extremely low, liver damage is usually extensive and the prognosis unfavourable. Persistently low cholesterol ester concentration, or a low ester/total ration, indicates continuing liver damage; a rise in the cholesterol ester often heralds improvement (Jones, 1942; Man, Kartin, Durlacher and Peters, 1945; Thannhauser, 1947).

After an intravenous injection of the dye bromsulphthalein, excretion of the dye takes place almost entirely via the liver. It is probably the most efficient and satisfactory artificial medium to test the excretory capacity of the liver when jaundice is absent (Teitelbaum, Curtis and Goldhamer, 1945). The test measures the retention of dye 45 minutes after the injection of 5 mg./Kg. body weight

of bromsulphthalein.

(c) Estimation of the synthesising and conjugating powers of the liver.

The liver removes noxious materials or renders them harmless by various processes - by oxidation or reduction, excretion, or by conjugating the toxic substances with amino-acids, glucuronate, inorganic radicals such as sulphate. A convenient function test (Quick, 1933) depends on the conjugation of intravenously introduced sodium benzoate with glycine in the liver to form hippuric acid, the excretion rate of which can easily be measured in the urine. Provided renal function is normal, liver function can be determined.

(d) Estimation of the total plasma proteins and of their partition into albumin and globulin.

Composition of the plasma proteins is affected quantitatively and qualitatively in liver disease. Most of the albumin and at least a small fraction of the globulin are synthesised in the liver. A fall in the albumin and a rise in the globulin, especially the gamma globulin, occurs in cases of liver damage. Both qualitative and quantitative changes in the plasma albumin and globulin determine

the results in a number of empirical tests in which various colloidal substances are thrown out of solution when sera from cases of hepatic disease are mixed with the test reagent. These abnormal results are chiefly due to changes in the gamma globulin, but also to some alteration in substances present in normal serum which would inhibit the reaction; these inhibitors reside mainly in the albumin fraction, which is often greatly reduced. The chief tests in this group are the cephalin-cholesterol flocculation test, the colloidal gold reaction and zinc sulphate reaction, all being reactions with gamma globulin; the thymol turbidity and thymol flocculation tests, in which, in addition to gamma globulin the lipo-proteins are concerned. Because serum proteins are altered in many diseases, such tests are not specific for liver disease (Franklin, Popper, Steigmann and Kozoll, 1948; Popper, Steigmann and Szanto, 1949). They are of value, however, in differentiating cellular from obstructive hepatic disease and employed with appropriate reservations, they are of some assistance in following the progress of liver disease.

(e) Estimation of the amount of prothrombin in the blood before and after the administration of vitamin K.

A low prothrombin in the presence of an adequate amount of vitamin K indicates the failure of the liver to synthesise prothrombin. A low prothrombin concentration in the absence of jaundice usually indicates serious liver damage, and failure of response to large doses of vitamin K given intramuscularly or intravenously confirms the presence of severe liver damage (Popper and Schaffner, 1950). It is also true if jaundice is present.

(f) Estimation of the amount of urobilinogen excreted in the urine.

Urobilinogen is normally formed in the bowel from bilirubin by bacterial action. Normally, all that is absorbed from the intestinal tract is excreted by the liver, only 0 - 0.4 mg. appearing in the urine in 24 hours (Steigmann and Dyniewicz, 1943). An increase in urinary urobilinogen occurs with an impaired liver, a partial obstruction of the common bile duct or it may be due to an overloading of the liver as

a result of increased urobilinogen production following haemolytic disease. If the common bile duct obstruction is complete, no bilirubin enters the intestinal tract, no urobilinogen is formed and none is found in the urine or faeces.

The various tests of liver function given are in general likely to yield positive evidence of impairment in conditions of diffuse generalised damage to the organ and they are often of value in following the prognosis of a case. Owing to the substantial functional reserve of liver tissue, it is not uncommon to obtain results within normal limits for the various tests even when there is quite extensive infiltration of the organ due to malignant deposits or other substances. On the other hand, by a careful selection of the above tests, Rennie (1947) found it possible to distinguish diffuse affections of the liver cells from other causes of hepatic enlargement. After one has obtained information from the various tests it may still be necessary to carry out a liver biopsy, in which a small piece of liver tissue is obtained by puncture of the liver with a

trocar and subjected to histological examination. The method is not without risk and the information gained may be misleading if an unrepresentative piece of liver is obtained. A biopsy taken at a laparotomy is much more satisfactory since a representative piece of liver may be taken for microscopic examination.

4. EMBRYOLOGY.

The following brief account of the embryology of the liver is as usually described in the standard text-books on anatomy, based on the earlier workers Kolliker, (1880) and Choronshitzky (1900) as well as the classic studies of Bloom, (1926). The liver develops by proliferation of cells from the blind ends of a Y-shaped diverticulum which grows from the endoderm of the foregut of the embryo into the septum transversum. The pericardium and diaphragm develop from the cranial portion of the septum transversum. The caudal portion becomes the ventral mesogastrium in which the vitelline veins anastomose freely to form a venous plexus. The proliferating liver cells grow into the caudal portion of the septum transversum, and invaginate into the venous walls, and grow freely in the blood spaces. By continued growth, the liver cells eventually break

the blood spaces into the sinusoids of the adult organ. In the adult liver the blood in the sinusoids is thus in direct contact with the liver cells. The original diverticulum from the foregut becomes the common bile duct and the Y-shaped bifurcation becomes the hepatic ducts. A blind diverticulum from the common bile duct becomes the gall bladder and cystic duct. The hepatic ducts divide and redivide until the liver cells grow from the blind end of each into the blood present in the venous plexus formed by the vitelline veins.

Embryologically, the centre of each lobule is a bile duct, but this is not the histological structure of the adult lobule. The lobules of the embryo fuse and are subdivided by the growth of fibrous septa along the bile ducts which lie at the periphery of the adult lobule.

Hans Elias (1955a, 1955b, 1955c) examined the livers of 30 species from 16 orders of 8 classes, and came to conclusions regarding the development of the liver that differed considerably from many previous workers. His views on the development of the liver in mammals can be stated as follows :-

From the wall of the liver diverticulum cords and ridges of cells proliferate. These cells

invade the mesenchyme of the septum transversum and surround the pre-existing sinusoids. The invading cells migrate round these sinusoids, and unite to form plates which are at first several cells thick. The sinusoids arise at first as small isolated vesicles and unite secondarily to form a network which joins the omphalomesenteric and umbilical veins. The phenomenon of "intercrescence" seems to occur only in the later stages (5-10mm embryo) in a restricted area of the already formed liver. Elias made an observation that had not been made before, namely, that near the omphalomesenteric veins there is taking place an intense proliferation of the visceral mesothelium. The mesothelial cells invade, interstitially, the dorsolateral parts of the septum transversum and invert the blood vessels in the same manner as the entodermal liver cells do. Both kinds of cells unite with each other to form liver plates which are at first 3 - 5 cells thick.

The entodermal contribution, however, is much more extensive than the mesothelial contribution. In an human embryo of 19 somites stage the early liver consists of a large unpaired ventrocranial

portion which is entirely of entodermal origin; it has, further, 2 slender caudo-lateral lobes which flank the enteria intestinal portal. The tapering, paired, caudal ends of the liver anlage are purely mesodermal. Between the ventral anterior entodermal and posterior mesodermal parts of the human liver anlage, there is paired intermediate zone of mixed origin.

The liver cell plates which are originally 3 to 5 cells thick begin to thin out at the 27 somite stage (it may, however, occur earlier or later). They finally become one cell thick, possibly due to the stretching effect of the fully engorged sinusoids.

With regard to the duct system, Elias does not believe there is any evidence of evagination or sprouting of the duct system from the gut. The duct system he believes, arises within the liver and subsequently joins the gut. In man it arises by differentiation from the hepatic cells.

The embryos studied by Elias indicated many different ways of development. There were, for instance (a) variations in the embryonic plate thickness, (b) the origin of the duct system varied from species to

species, (c) there were several modes of entodermal and mesodermal duct development, and (d) different sources of the entoderm and mesoderm contributed to the embryo liver. Altogether he found 12 different modes of liver development, and he emphasised the fact that the minute histology of the organ is determined, not by the material from which it is built, but by the toxonomic position of its bearer.

The similarity of the structure of the livers of all vertebrates has been noted by Elias and Bengelsdorf (1952) and Bagley and Grafflin (1952). These workers noted that plate thickness and the mode of ending of the bile canaliculi were the only detectable differences of liver histology among the vertebrates, and they stressed the uniformity of liver structure throughout the phyllum of the vertebrates was probably greater than that of most organs. (Figures 1 - 4).

Elias (1955) therefore, thought it justifiable to stress the similarity of the liver of vertebrates. He stated that "the liver of all vertebrates (except that of the adult lamprey) is a continuous mass of cells tunnelled by a labyrinth of lacunae in which the sinusoids are suspended. In other words, the liver

Figure 1.

A stereogram based on the histological description of the liver of lower vertebrates.

(Taken from Research in the Service of Medicine, Volume 37, G.D. Searle and Company, Ltd.)

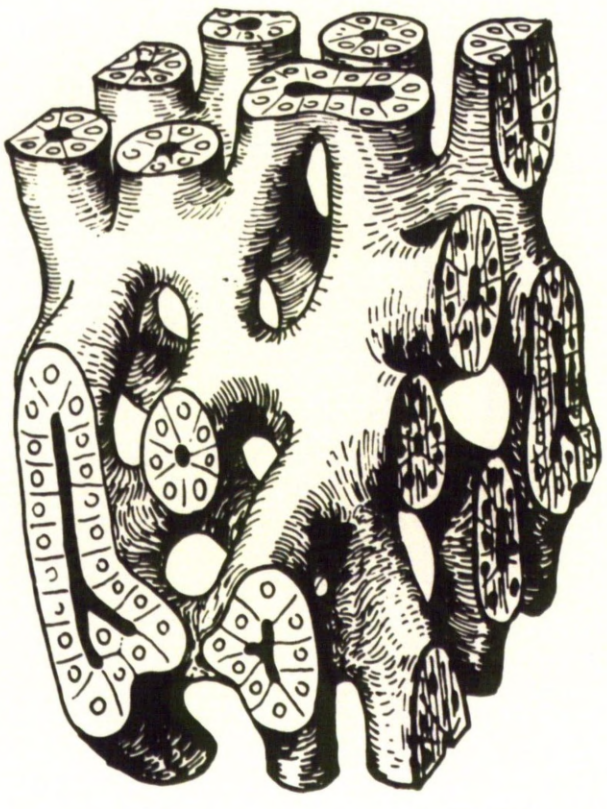
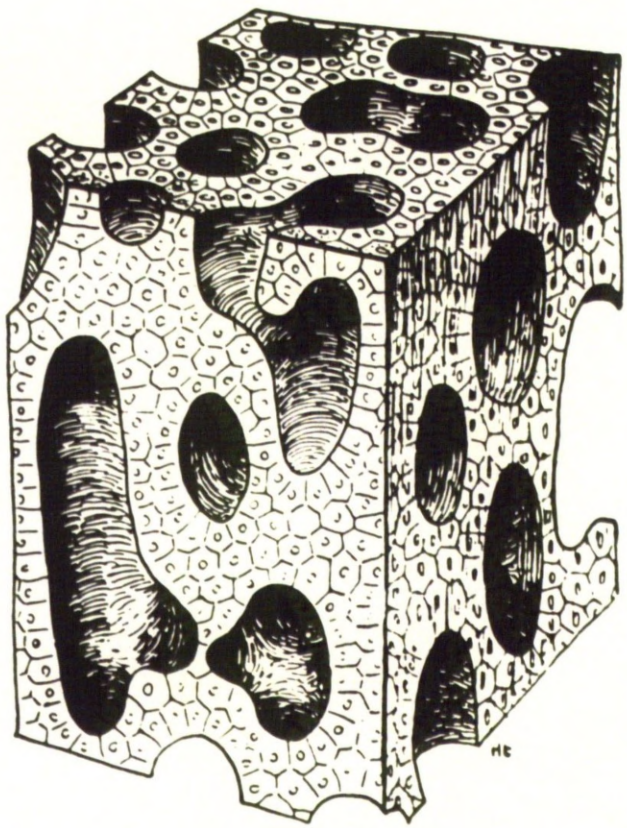


Figure 2.

A stereogrm of the true structure of the liver of lower vertebrates.

(Taken from Research in the Service of Medicine, Volume 37, G.D. Searle and Company Ltd.)



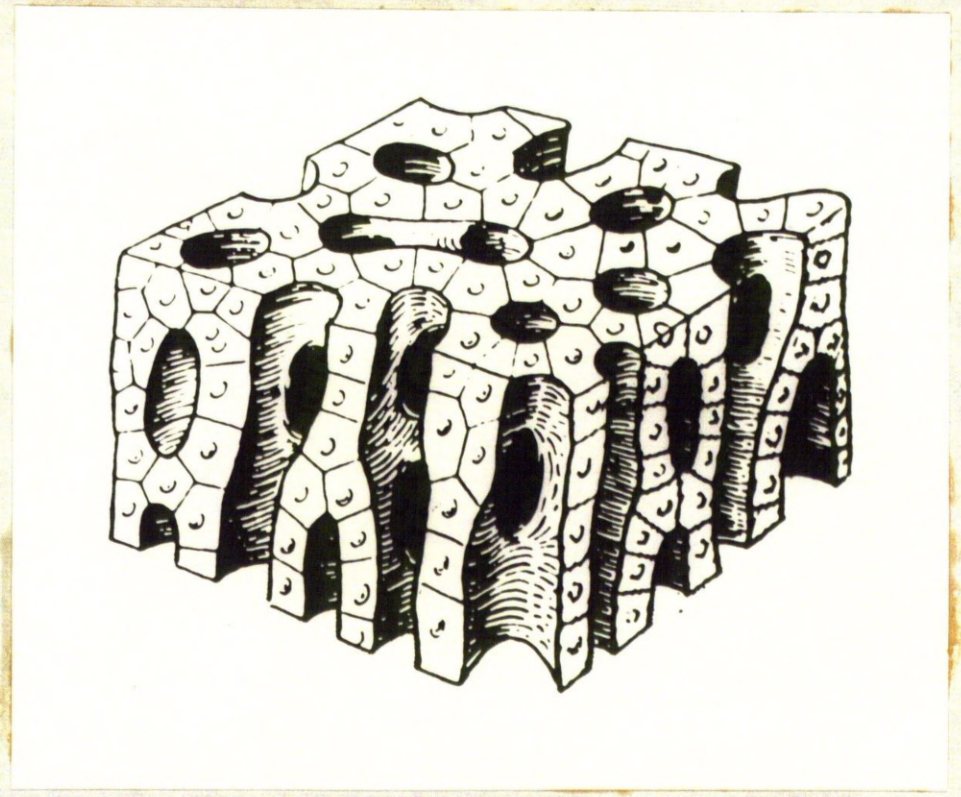
Figures 3 and 4.

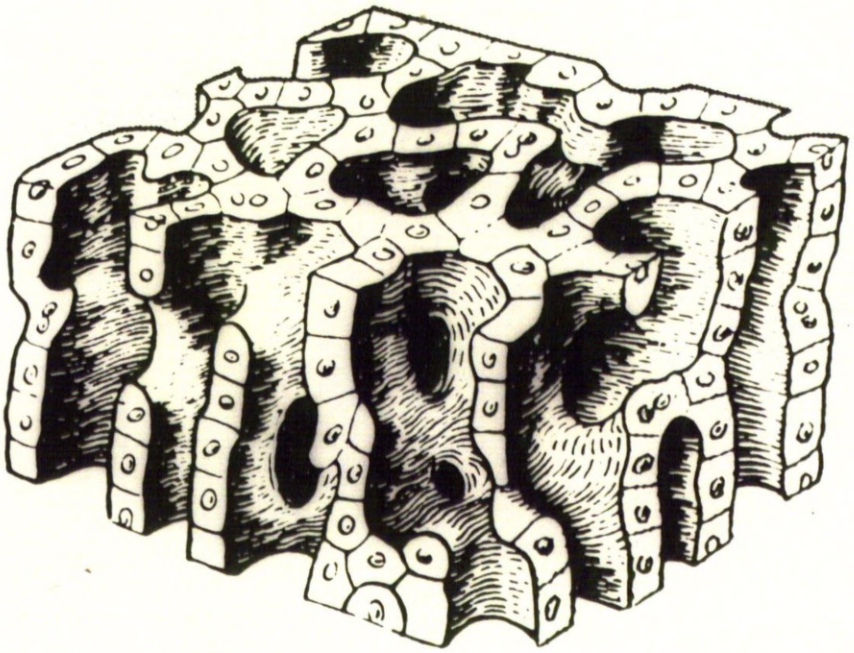
The basic types of liver among the vertebrates. Figure 3, shows the tubulosinusoidal type with narrow, cylindrical lacunae (rabbit and horse) and Figure 4, shows the sacculosinusoidal type with wide, irregularly-shaped lacunae (duckbill, spiny anteater, cat and man.

The liver of the dog appears to be of an intermediate type.

The distinction between the two types may be of physiological importance since it determines the ratio of blood volume to hepatic cell volume as well as the relative size of the surfaces of liver plates exposed to the blood-stream and the relative blood storage capacity of the liver.

(Taken from Research in the Service of Medicine, Volume 37, G.D. Searle and Company, Ltd.)





parenchyma is a muralium* of cells (i.e. an irregular wallwork of cells). In all vertebrates the liver is interposed into the return blood flow from the intestine".

5. MICRO-ANATOMY OF THE LIVER.

The hepatic cell is the smallest unit of the liver, and it performs most of the numerous functions of the liver. This cell has a rather simple structure despite its extremely varied functions. It has the general characteristics of parenchymal cells as well as morphological and functional features peculiar to the liver. The cell is polygonal in shape having eight or more sides. It contains a nucleus, nucleoli and cytoplasm as other cells, but does not have a specific cell membrane, the condensed cytoplasm at the periphery of the cell acting as one. (Maximow and Bloom, 1948).

More than a hundred years ago, Gerlach presented the concept that hepatic cells are arranged in the form of plates or cords (Figure 5) and this was illustrated by

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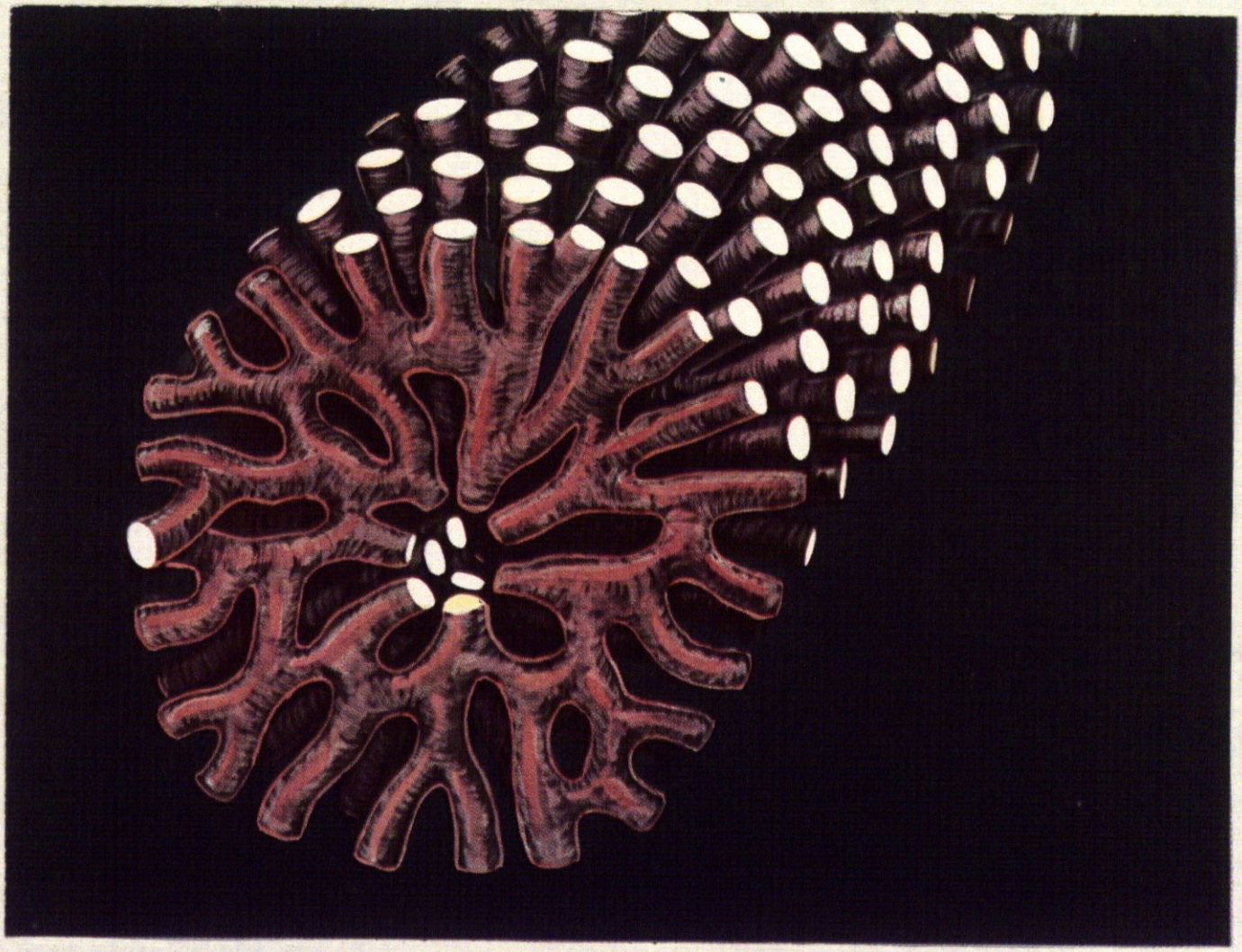
* Elias was the first to use the word 'muralism' in his booklet 'Functional Morphology of the Liver' published by G.D. Searle & Co., Chicago, 1953. He pointed out the word does not exist in Latin but is derived from the Italian 'muraglia'.

Figure 5.

A stereogram illustrating the classical concept of the liver lobule as consisting of cords.

(Taken from Research in the Service of Medicine, Volume 37, G.D. Searle and Company, Ltd.)



Stohr and Braus. Between these cords of cells the blood in the sinusoids flows from the interlobular branches of the portal vein and hepatic artery to the central veins (the smallest branches of the hepatic veins). The blood is separated from the liver cells by the sinusoid endothelium. Some of the cells in the endothelium, the Kupffer cells, are more differentiated than the others, and they have a great phagocytic capacity. They often bulge out into the lumen of the sinusoids and also differ from the rest of the endothelium by having a larger nucleus and many phagocytosed particles within their cytoplasm. The cytoplasm of the polygonal hepatic cells contains many granules and vacuoles. The appearance, as with all cells, is largely dependent upon the fixative used and some of the granules may be artefacts, especially with the routine histological fixatives. Within the cytoplasm are irregularly arranged rod-shaped or granular mitochondria. The Golgi apparatus has been stated to be related to the bile canaliculi (Pfuhl, 1932), and its function is apparently concerned with the formation of bile and some of the enzymes of the liver. In the cytoplasm, fat droplets and glycogen also occur. One or more nucleoli are contained within the nucleus of

the hepatic cell. The polygonal hepatic cells are plastic, being able to vary their shape with the different pressure occurring in the sinusoids. (Brugsh, 1932).

The histological appearance of the hepatic cells varies at different times, particularly with regard to the stored substances. The glycogen content depends upon the sex of the animal, nutrition and composition of the preceeding diet (Kerly and Ottaway, 1954). The glycogen distribution also varies in different zones of the liver lobule (Gomori and Goldner, 1947) and the content is reduced with starvation (Ekman and Holmgren, 1949; Aterman, 1952). The fat droplets in liver cells only disappear late in starvation (Pfuhl, 1932). Also functional variations in the appearance of mitochondria have been described (Kater, 1931; Deane, 1942; Paigen, 1954).

The original theory, that the arrangement of hepatic cells simply as a double row of liver cells surrounded (Figure 6) on all sides by blood sinusoids and radially arranged about a central vein, has been accepted since it was first presented. Almost a century ago, Hering,(1872) assumed that the liver of the rabbit was a continuous mass of cells traversed

Figure 6.

A stereogram illustrating the old theory of cords being two cells thick and wrapped in reticuloendothelium.

(Taken from Research in the Service of Medicine, Colume 37, G.D. Searle and Company, Ltd.)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by the sinusoids so that one-cell-thick plates are formed, but this theory has been neglected except for an occasional reference. The structure of the mammalian liver has recently been re-examined by Elias (1949, a.b.) on the basis of three-dimensional reconstructions. He claims that the liver is a continuous mass of hepatic cells which are normally arranged in the form of plates (laminae hepatis), one cell in thickness (Figure 7), which form the wall of spaces called hepatic lacunae. Through perforations in the liver plates, the lacunae are connected with each other and form the hepatic labyrinth. Thus the plates form an irregular wallwork which surrounds spaces containing the sinusoids. The shape and size of the polygonal hepatic cells varies depending upon the position of the cell within the plates. The histological appearances of the liver are more readily correlated with this view (Figures 8 and 9), rather than the older concept of the hepatic cell arrangement. The hepatic lobules are ill-defined areas which surround the central veins and are continuous with each other. The liver parenchyma is tunnelled by portal canals (Figures 10 and 11) or tracts which contain a branch of the portal vein, hepatic artery and bile duct, lymphatics

Figure 7.

A liver plate, one cell thick, richly perforated and connected with other liver plates.

(Taken from Research in the Service of Medicine, Volume 37, G.D. Searle and Company, Ltd.)

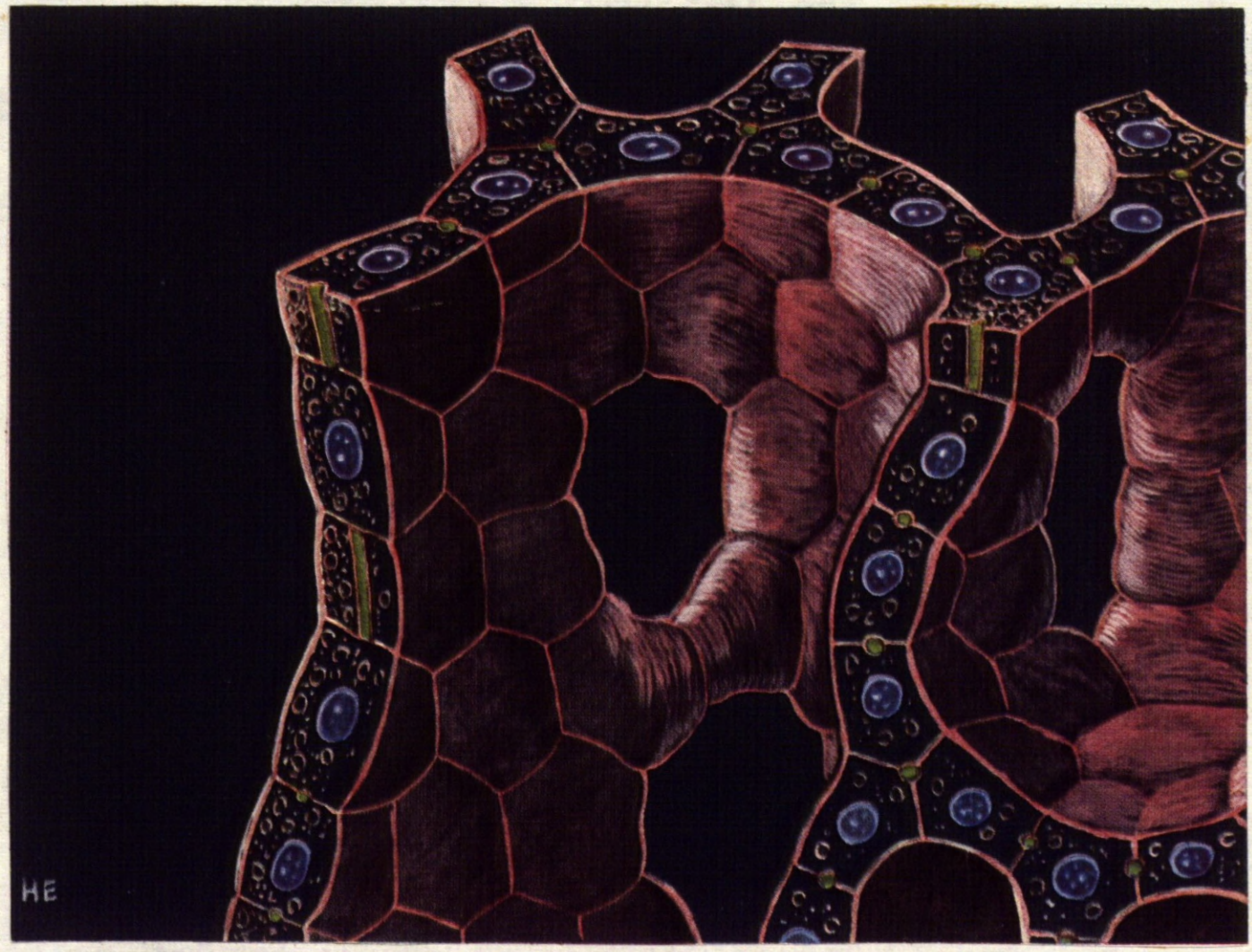
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Figure 8.

A section of the human liver showing unending rows of cells, one cell wide.

(Taken from Research in the Service of Medicine, Volume 37, G.D. Searle and Company, Ltd.).

Figure 9.

Block of human tissue, the upper surface is identical with that of Figure 8.

(Taken from Research in the Service of Medicine, Volume 37, G.D. Searle and Company, Ltd.).

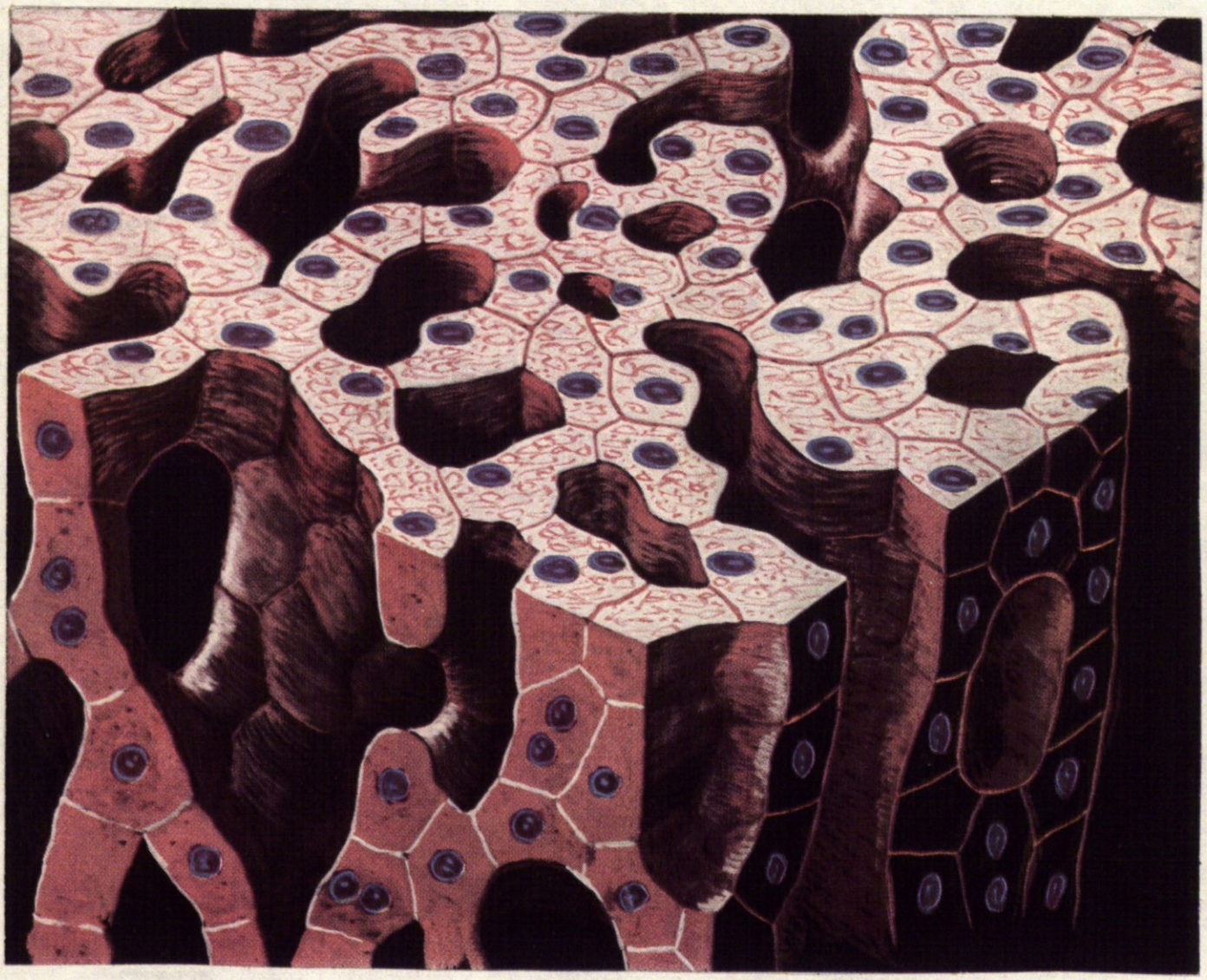


Figure 10.

A stereogram of a portal canal of the dog.
Portal vein, inlet venule and sinusoids are in violet; artery and arterial capillaries in red; ducts in green. The portal canal is a tunnel surrounded by the limiting plate.

(Taken from Research in the Service of Medicine, Volume 37, G.D. Searle and Company, Ltd.)

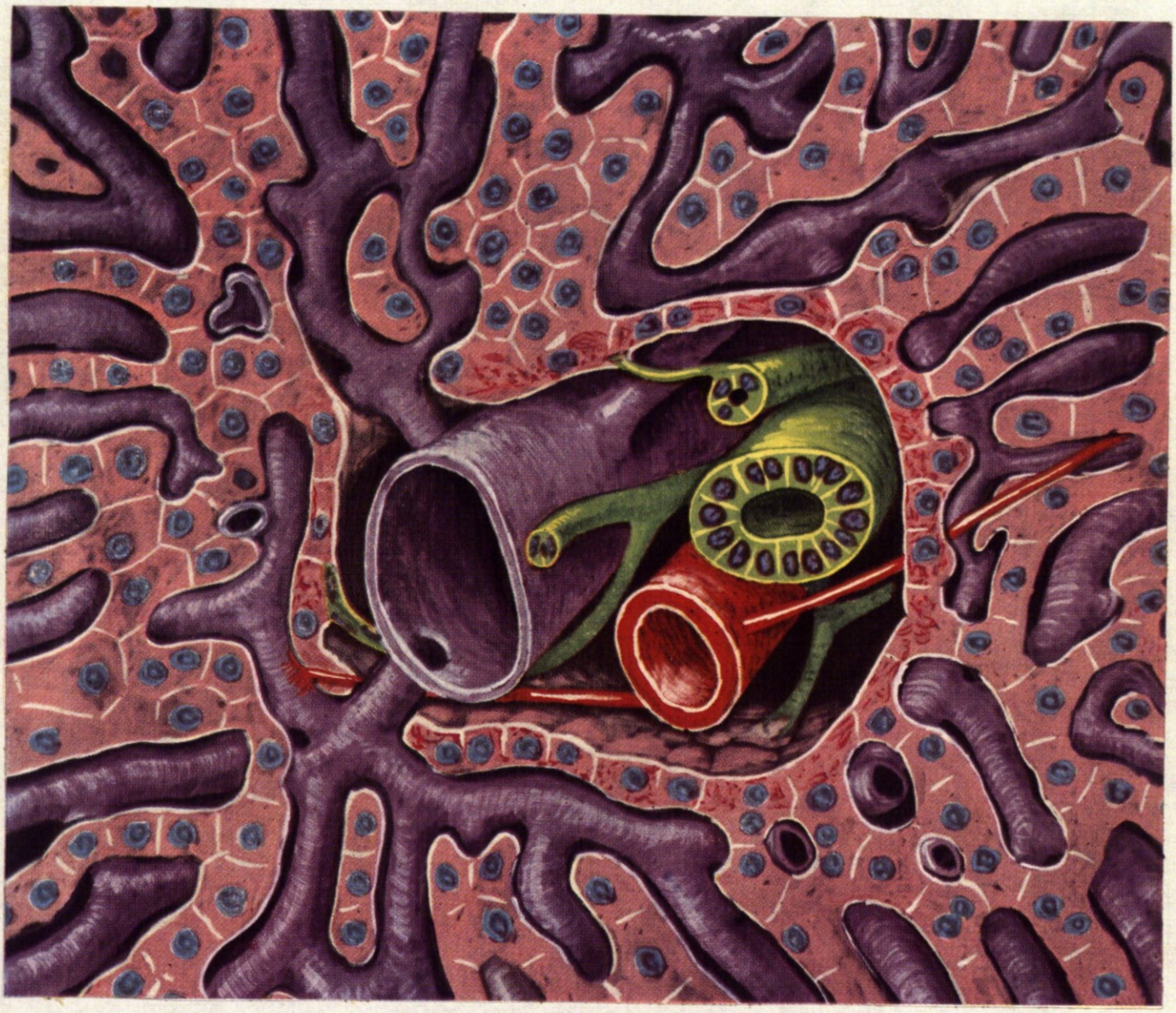
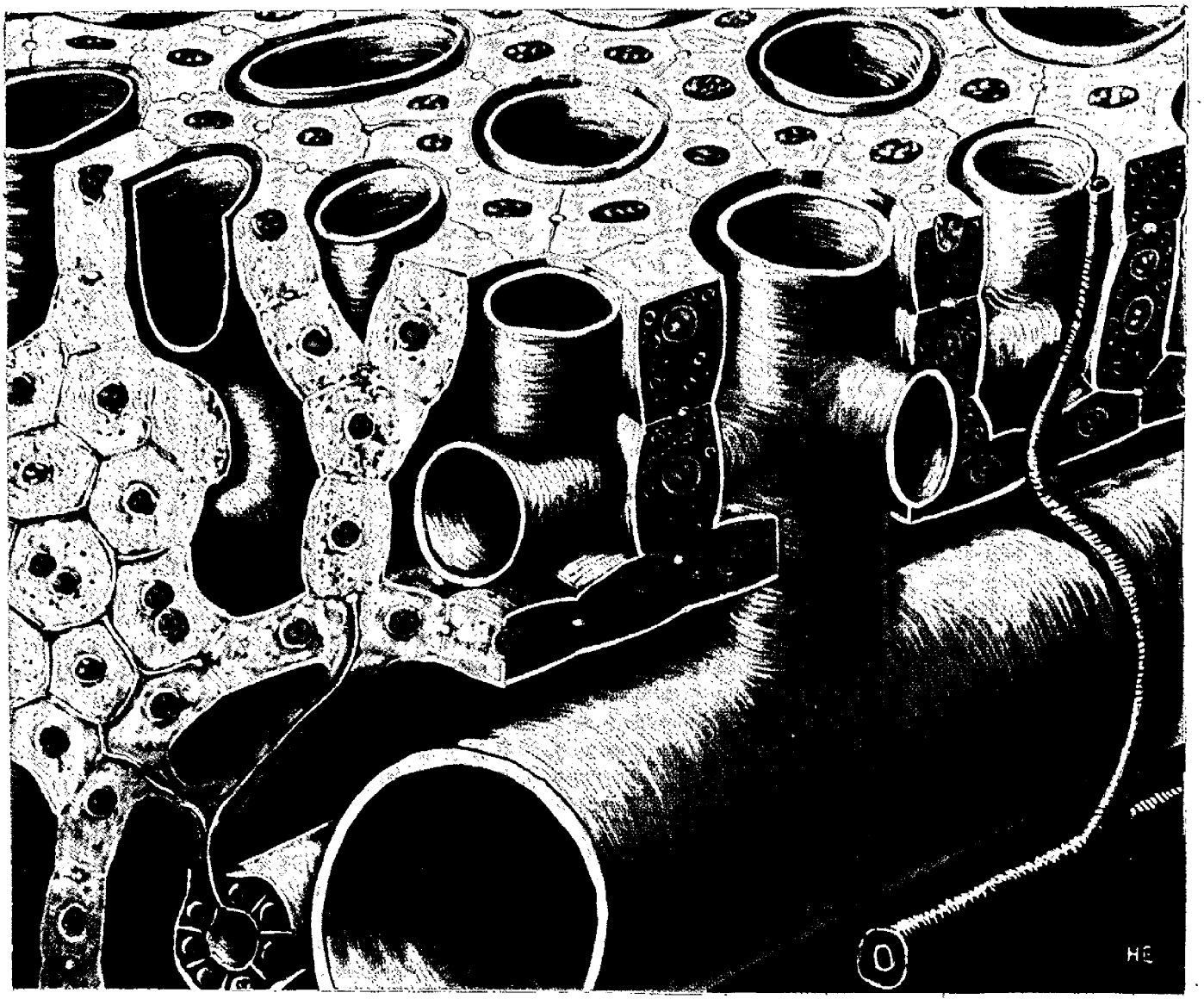


Figure 11.

A stereogram of portal canal showing :- artery and intralobular arterial capillary, inlet venule, distribution of the bile canaliculi and the connection of the limiting plate through the cholangioles with a bile duct.

(Taken from Research in the Service of Medicine, Volume 37, G.D. Searle and Company, Ltd.).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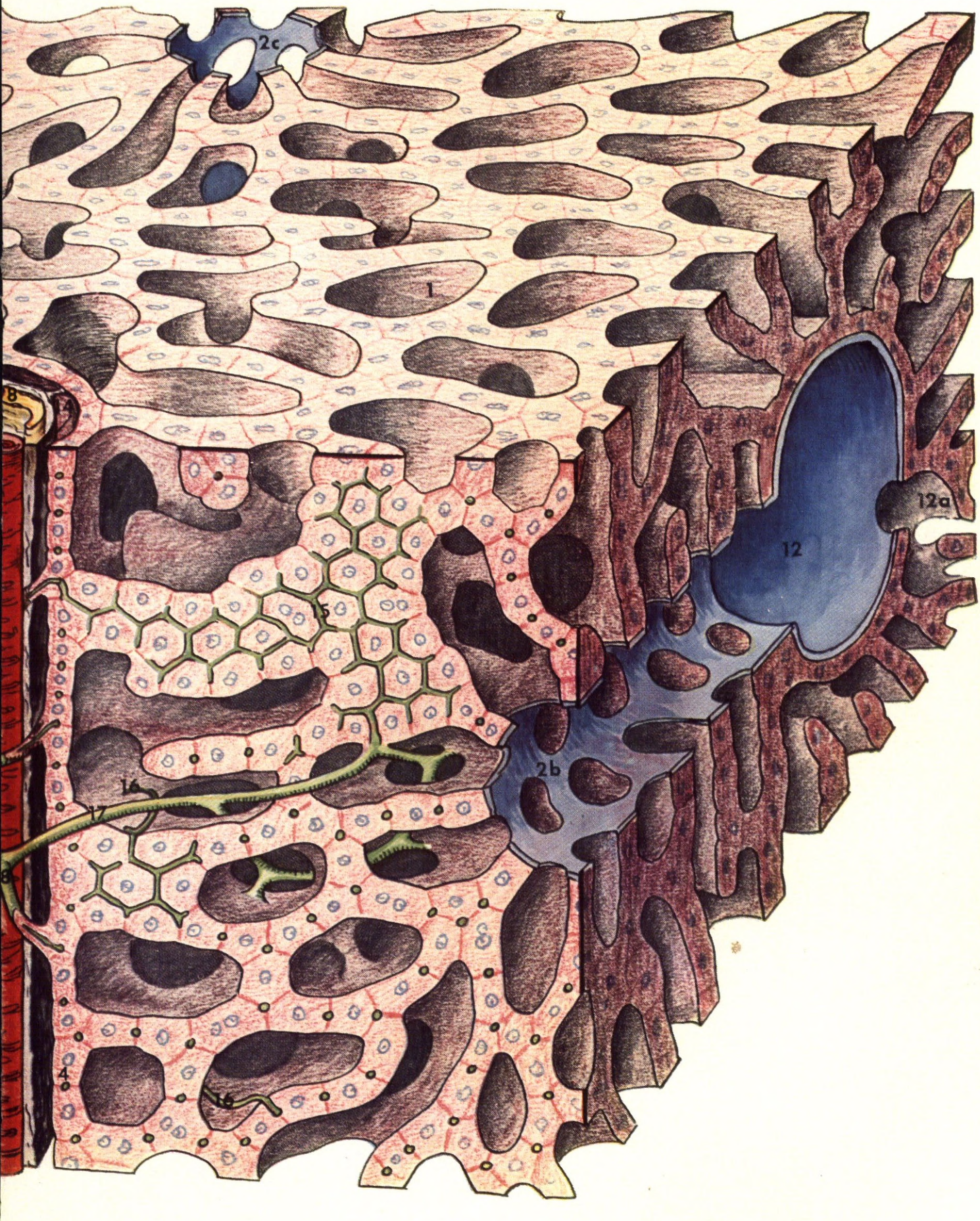
and connective tissue. The hepatic cells are arranged in the form of a one-cell-thick plate, as a sheath enveloping the portal canal (Figure 12). This sheath is called the 'limiting plate' and it is continuous with the intra-lobular plates of cells. From the portal vein, venules penetrate through holes in the limiting plate and ramify into sinusoids. From the hepatic artery two sets of arterial capillaries pass into the sinusoids . A third set may also be present. Each set of arterial capillaries has sphincters. Minute bile ducts enter the lobules and receive bile from intra-lobular plates. The bile ducts never end freely, always forming loops or networks.(Figure 14). The lymph ducts of the liver have been shown by retrograde dye injection (Bollman, 1950) to follow the branches of the portal vein, the hepatic artery and the hepatic veins.

Blood supply.

Although the blood supply of the liver varies to some extent with the animal species it is felt necessary to describe the vascular supply within the liver of the higher species (i.e. in those groups of animals including man where the hepatic blood is re-



9 periportal connective tissue
10 inlet venules
11 sinusoids
12 sublobular vein
12a lacuna connecting with sublobular space
13 perisinusoidal space of Disse
14 periportal space of Mall
15 bile canaliculi within liver plates
16 bile canaliculi on the surface of liver plates (not frequent)
17 intralobular cholangiole
18 cholangioles in portal canals

Figure 13.

A reconstruction from serial sections showing the intralobular arterioles and arterial capillaries. These empty into the central sinusoids. The arterial capillaries have sphincters. The intralobular ducts form loops.

(Taken from Research in the Service of Medicine, Volume 37, G.D. Searle and Company, Ltd.)

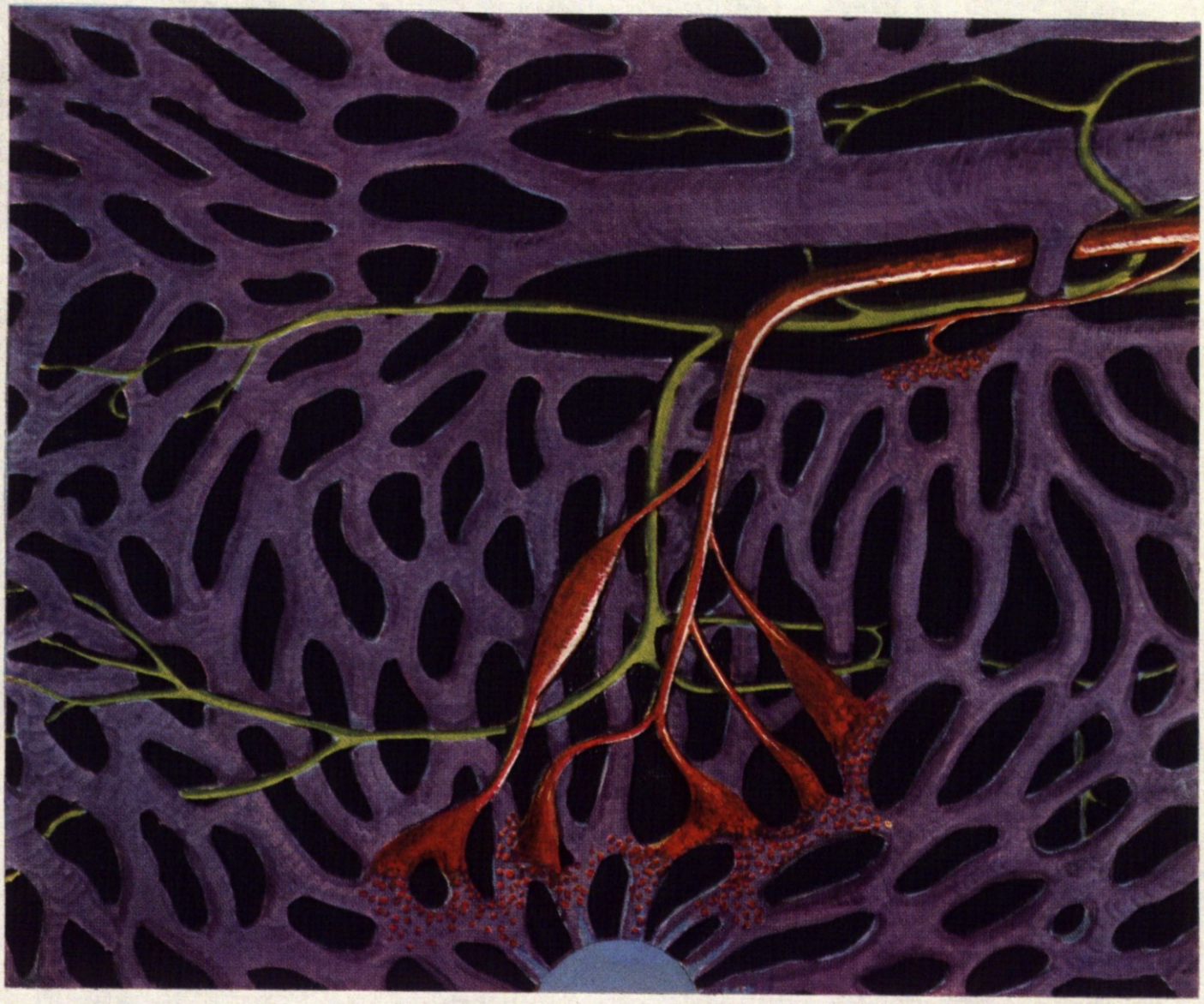
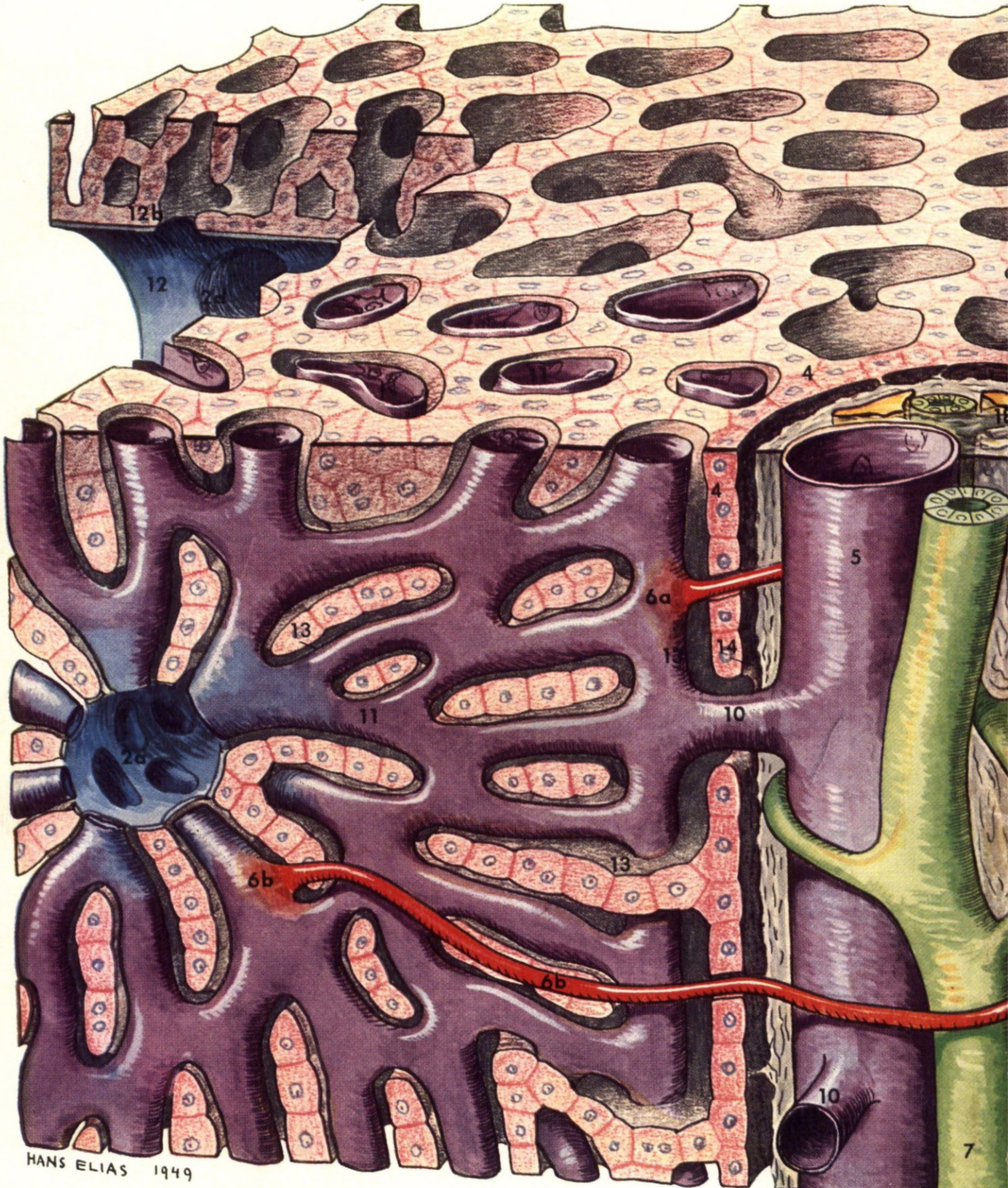


Figure 14.

A diagram of intrahepatic portal branching.

(Taken from Research in the Service of Medicine, Volume 37, G.D. Searle and Company, Ltd.).



FRONTISPIECE / SYNOPSIS OF THE STRUCTURE OF THE NORMAL HUMAN LIVER:

1 lacuna
2a, b, c, d central veins
3 portal canal
4 limiting plate
5 portal vein
6 hepatic artery
6a arterial capillary emptying into paraportal sinusoid
6b arterial capillary emptying into intralobular sinusoid
7 bile ducts
8 lymph vessel

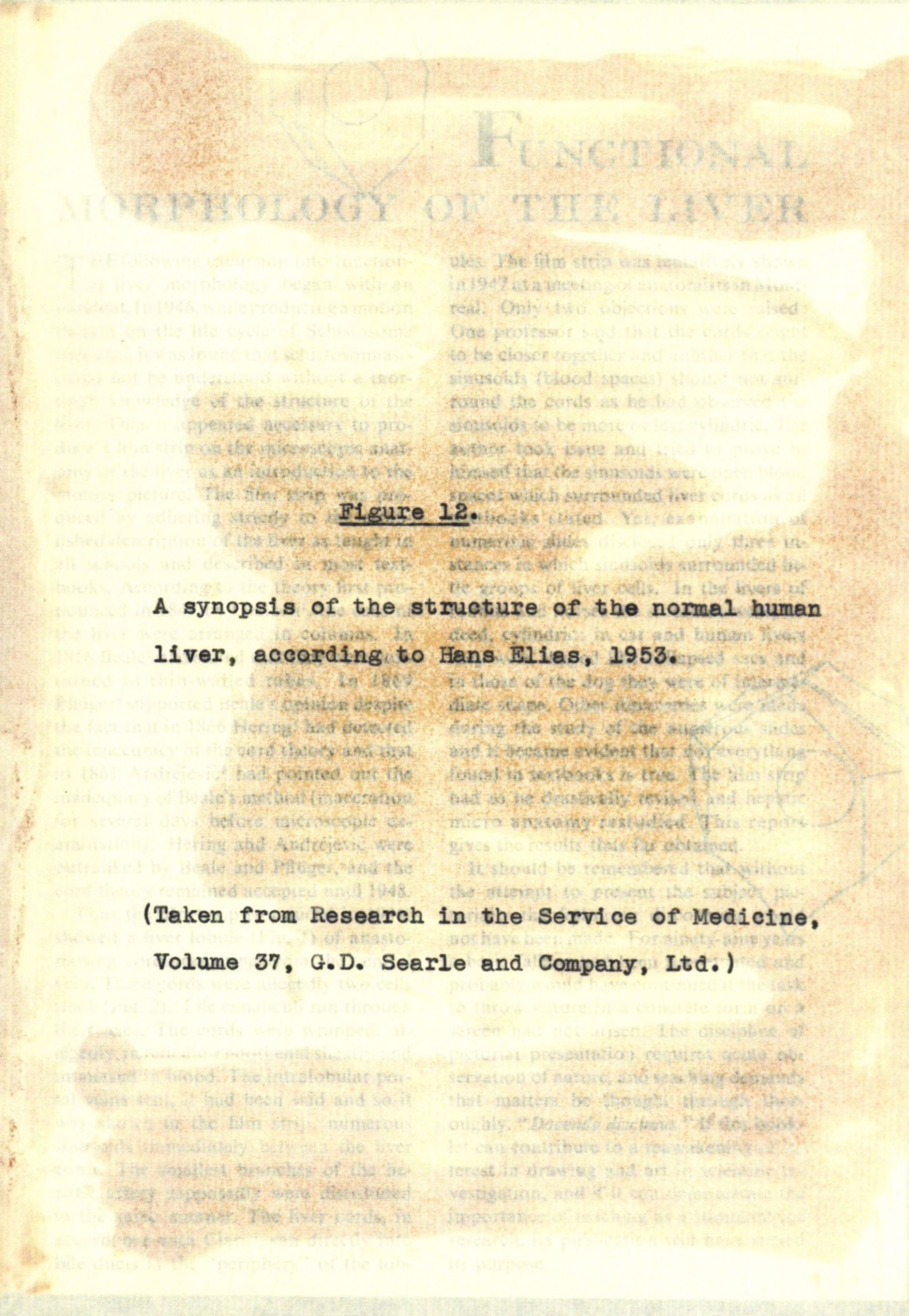


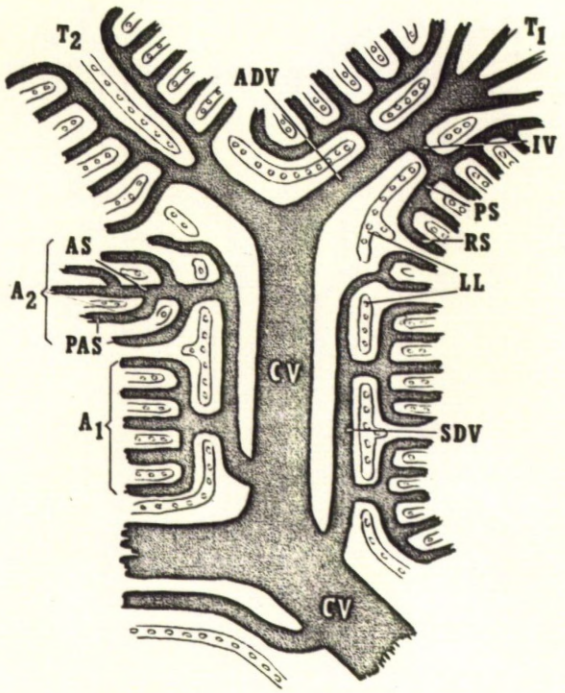
Figure 12.

A synopsis of the structure of the normal human liver, according to Hans Elias, 1953.

(Taken from Research in the Service of Medicine, Volume 37, G.D. Searle and Company, Ltd.)

Diagram of intrahepatic portal branching:

A_1 arborization type I
A_2 arborization type II
ADV axial distributing vein
AS axial sinusoid
CV conducting vein
IV inlet venule
LL lamina limitans
PAS paraxial sinusoid
PS peripheral sinusoid
RS radial sinusoid
SDV small distributing vein
T_1 terminal twig type I
T_2 terminal twig type II



ceived from two sources, the portal vein and the hepatic artery).

The Portal Venous Supply.

Wakim and Mann (1942); Wakim (1954); and Elias (1955d) describe the portal venous supply to the liver in mammals (Figure 15) as follows :-

'After the portal vein has divided into the main branches and consecutively into smaller branches, eventually small portal tracts are reached in which a central distributing vein, less than 0.3 mm in diameter gives off short inlet venules at right angles. Finally, the smallest portal vein divides into two terminal twigs which enter the parenchyma. The inlet venules extend through the limiting plate into the peripheral sinusoids of the liver units (or lobules) supplying the bulk of the portal blood to the parenchyma. From the peripheral sinusoids blood flows through radially arranged sinusoids to a central vein. Branches of the portal vein above 0.3 mm do not discharge inlet venules directly into the sinusoidal system and have been called conducting veins by Elias (1955d). These veins lie in the larger portal tracts and

are accompanied by parallel-running, small distributing veins which carry blood sometimes in the same direction, and sometimes in the opposite direction to the current in the larger conducting vein.

The blood supply and draining of the structures in the portal tract, especially of the bile ducts, differ from those of the hepatic parenchyma in that the portal vein branches act as a blood-draining rather than a blood-supplying vessel. Small venules collecting the blood from the capillary plexus in the portal tracts, and especially around the bile ducts, transport it into the lobular parenchyma by uniting with the inlet venules, acting as 'internal roots' of the portal vein'.

Hepatic Arterial Supply.

The portal vein carries a large volume of blood to the liver at a low pressure, the hepatic artery carries a small volume of blood at great velocity and at a much higher pressure (Wakim 1954). The hepatic artery ramifies in parallel with the portal vein branches. Terminal arterioles release blood into the

lobular parenchyma and terminate at different levels of the lobule, thus providing fresh arterial blood to all its parts. The bulk seems to be released, however, in the periportal area by short arterioles. The arterial branches in the portal tract also supply the peribiliary plexus (Maegraith 1958), whence the blood is drained to the 'internal roots' of the portal vein.

The Sinusoids.

Wakim and Mann (1942) as a result of their trans-illumination studies of living mammalian liver described the sinusoids as ramifying in a radial manner between columns of parenchymal cells. They noted that the sinusoids which drain into the ramifications of the hepatic veins were slightly ampullated just before they joined the draining vein, and that they were narrowed at their point of junction with the vein and this appearance gave the impression of a sphincter between sinusoid and central vein. They observed also that the sinusoids appeared to undergo narrowing at their points of origin from the main portal vessels.

Maegraith (1958) using a similar technique confirmed these findings and extended them, but his

description of the sinusoid differed from Wakim and Mann (1942). The sinusoid was stated to be a cylindrical tube continuously lined by endothelium and with no Kupffer cells bulging into the lumen. He noted that the sinusoids branched and anastomosed freely, but did not consider that they 'radiated" from the central vein. Rather, he reported that the sinusoids tended to form large 'collecting sinusoids' which emptied into the central vein or into the larger radicles of the hepatic veins. Some sinusoids showed minimum branching and anastomosis and appeared to run almost directly from the portal venous to the hepatic venous side. The sinusoidal pathways were usually separated by a single layer of epithelial cells. He did not see the 'spaces of Disse' on any occasion. In confirmation of the work of Rappaport, Borowy, Lougheed and Lotto, (1954) Maegraith found the drainage of the hepatic vein was not strictly lobular but involved sinusoidal elements from several adjacent portal tracts. Some sinusoids which arose from a single portal tract drained into several central or sublobular veins. The origin of blood reaching the sinusoids can be described according to Maegraith, (1958); Maegraith,

and Wenyon (1959), as follows :-

(a) Mixed Arterial and Portal Venous Blood

The hepatic arterial blood is mixed with portal venous blood through direct connections with the portal vein before the origin of the sinusoids, and also through the extensive bile duct.plexus, from which vessels proceed directly to the sinusoids.

(b) Portal Venous Blood

Sinusoids are supplied directly from small branches coming at right angles from the main trunk or from others which course along the portal tracts before entering the lobules. Terminal branches of the portal vein break up directly into sinusoids. There is a peripheral network of large sinusoids which arises from these branches of the portal vein and which appears to be separated from the portal tracts by a single layer of epithelial cells.

(c) Hepatic Artery.

In general, arterial branches anastomose with one another and portal venous branches do not. Arterial blood goes directly to the sinusoids from vessels in the portal tract. Intralobular arterial vessels which enter lobules before breaking up into sinusoids have not been observed.

The structure of the sinusoids of the liver

The sinusoids of the liver have been described above as having either an 'endothelial' lining or a lining of Kuppfer cells, or a lining which is a mixture of both (Figure 16). Certain workers, however, held that there was no true lining, that the liver cells were, in fact, directly bathed in blood. Hering and Simpson (1906), for example, observed in sections of canine liver, red blood cells and haemoglobin crystals inside the parenchymal cells and concluded that the endothelial lining of the sinusoids was, at least in part, incomplete to allow the passage of such large particles. Sharpey-Shafer (1929), as a result of injection studies, and the observation of histological sections, was firmly of the opinion that the sinusoids were without any endothelial lining of any sort. He did not think the Kuppfer cells sufficiently stable or immobile to constitute a 'lining'. He emphasised that the sinusoids communicated directly with the fine intracellular canaliculi of the liver cells, that is to say, the sinusoids were lined by liver cells themselves.

Electron microscopy has shown more clearly the relationship between the liver cells and the cells

Figure 15.

A stereogram showing the suspension of the sinusoidal network in the lacunae of the hepatic labyrinth.

(Taken from Research in the Service of Medicine, Volume 37, G.D. Searle and Company, Ltd.)

lining the sinusoids (Fawcett, 1955; Bennett, Huft and Hampton, 1959). The surfaces of the hepatic cells which face the sinusoids are often found to be covered with many short villous processes which are not seen with the light microscope. They are more variable in length and more disordered in their arrangement than the slender processes which comprise the striated border of intestinal cells, but in other respects are similar. Thick perinuclear portions of the sinusoid lining cells can be seen close to the hepatic cells in some places, but the thin peripheral extensions of the lining cells make up the bulk of the wall of the sinusoids. These extensions appear to rest lightly on the ends of the slender processes of the hepatic cells, without being firmly attached. This relationship of the lining cells to the microvilli of the hepatic cells, Fawcett believed, provided a basis for understanding the nature of the 'space of Disse' and its variable appearance as seen by the light microscope. If the surface processes of the liver cells vary in length in different functional states, then in some conditions the lining cells would appear closely applied to the parenchymal cells with no space of Disse visible; Whilst under other conditions, when

the processes were longer, the lining cells would seem to stand off a short distance from the surface of the liver cells.

In most places a thin layer of cytoplasm belonging to an endothelial cell of Kuppfer cell can be made out between the liver cell and the lumen of the sinusoid, but it is uncommon to find the small areas in which the villous surface of the liver cell appears to be in direct contact with the bloodstream, without the interposition of endothelium. Fawcett (1955) admitted that these apparent discontinuities in the wall of the sinusoid could be the result of shrinkage and retraction of the lining cells during fixation but he pointed out that buffered osmium does not have this effect on endothelium elsewhere. Fawcett (1955) and Bennett et al (1959) believed that the wall of the sinusoids may actually be discontinuous or fenestrated.

Fawcett noted that some of the lining cells contain phagocytosed material and others do not, but he does not think that the two categories differ sufficiently in their finer structure to justify regarding them as two distinct cell types.

Blood flow distribution within the liver

The blood flow through the sinusoidal bed of the liver does not follow a single well-defined pathway. Rapid and profound variations of the blood flow pattern in the sinusoids have been described. Using direct observations of the intrahepatic circulation in vivo, Wakim and Mann (1942) and Wakim (1954) described the circulatory activity of different regions of the liver. They noted that there was great variation in activity from one part of the liver to another, and even within the same lobule. In one lobule every sinusoid would be open, with blood passing through the lobule towards the central vein, and in another only a few sinusoids would be patent, while the majority were inactive. Some of these inactive sinusoids were observed to contain large numbers of red blood cells lying motionless within their lumina, whereas others appeared to be contracted and contained no blood cells.

Rapid changes in activity were also observed in a single lobule or a group of adjacent lobules. There were changes from inactivity, to partial activity and finally to full activity. In the phase of full activity, every sinusoid was open and blood flowed rapidly through the lobules into the central vein.

These alterations in the circulation were neither regular in occurrence nor did they take place in uniform sequence.

Intermittency of flow in the sinusoids of mammalian livers was also described by Wakim and Mann (1942) and Wakim (1954) when they observed a certain region of the liver for an hour or more, they noted that for several minutes the blood in one set of sinusoids coursed rapidly then gradually slowed for a while, only to increase again. In two or more adjacent regions, these changes in flow-rate occurred asynchronously and the shifts in rate in different groups of sinusoids exhibited no regularity or sequence. Maegraith (1958) was unable to confirm their findings of intermittency of sinusoidal flow as a normal occurrence. In Maegraith's opinion, intermittent flow only occurred in damaged livers. He did, however, admit that intermittent flow was occasionally seen in the normal liver in the very short vessels that connect one sinusoid to another.

Further evidence for circulatory variation within the liver has been given by the cine-angiographic studies of Daniel and Pritchard (1951). They emphasised the presence of innumerable intrahepatic

pathways of various lengths through which blood could pass from the portal vein to the inferior vena cava. They found that pathways that transmit blood through the parenchyma of the periphery of the liver are longer than those by which blood is carried through the parenchyma of the more central regions of the liver.

Two other important factors that may be concerned in the variability of the liver blood flow distribution have been suggested by Brauer, Leong, McElroy and Holloway (1956) and Brauer (1958). They described the hepatic circulation as being primarily a low pressure circulation and point out that in such a system mechanical disturbances which are of slight importance elsewhere can elict massive circulatory effect by "changing the critical closing pressure distribution of the component vessels". They have demonstrated the existence of such effects in the isolated, perfused rat liver. By increasing the portal pressure of such a preparation from 9 cm. to 12 cm. of water, they showed that the total blood flow rate was doubled, hepatic resistance was decreased by 50% and the number of perfused channels increased by about 100%. In the same preparation

they were able to show the importance of a second factor, namely tissue tension. They observed that a pressure of no more than 2 cm. of water exerted on the liver surface greatly increased the hepatic resistance to blood flow and completely stopped the bile flow. From these observations, Brauer and his associates concluded that "tissue tension and the effect of generalised, deforming pressures to which the liver is constantly subjected in the animal can indeed be important factors in the control of hepatic blood flow distribution".

In the same paper, Brauer (1958), after summarising the evidence and structural basis for the concept of intrahepatic circulatory variation, gave the following account of the arborisation of the vascular bed of the liver. He says -

> "There is a bewildering number of possible and substantially equivalent pathways through the sinusoidal bed, open to a particle of blood almost anywhere along its pathway through the liver. This property of the hepatic vasculature is associated with the fact that high order, extensively inter-anastomosed vessels of near capillary dimensions branch off from or lead

into vessels at almost all lower orders. Thus blood from a main portal branch may enter directly into the sinusoidal bed, or may proceed down an orderly progression of decreasing vessel sizes leaving these for the sinusoids at virtually any given level. To describe such a circulation, the classical pattern of more or less branched conduits aligned in linear order is impracticable. To approximate conditions in the liver a degree of arborisation is required which leads to an arrangement of wholly unmanageable complexity. A more promising model of somewhat statistical character, is that of a sponge with an array of hypodermic needles inserted so that their points form two interlocking lattices. If fluid is injected via the hydrodynamically joined needles forming one lattice, and withdrawn at an equal rate from those forming the second lattice, again joined hydrodynamically, a circulation results having many of the properties of the hepatic sinusoidal circulation. A particular property of such a system is its plasticity - the readiness with which the system will respond with a re-

distribution of flow to even slight regional disturbances. A similar plasticity is observed in the liver. In this connection attention should be called to the fact that the hepatic artery provides a potentially high pressure source of blood which in such a system may serve as a safety supply able to take over and to provide needed blood to regions from which the lower pressure circulation has been deflected by some disturbance".

The Hepatic Veins

The draining hepatic veins start with the central vein into which the sinusoids drain freely; there is no limiting plate of liver cells (Gans, 1955; Netter, 1957; Hobsley, 1958). The central veins unite to form sublobular veins and finally join the inferior vena cava.

The liver is considered capable of regulating the amount of blood that flows through it. Knisely, 1939, 1949 and Knisely, Bloch and Warner, 1945 among others attributed this to contractions in the sub-lobular veins, the interlobular vessels and sinusoids.

The blood flow through the sinusoids is intermittent and can be varied, being at times stationary in isolated sinusoids or larger parts of the lobules, a condition which is of functional significance (Wakim and Mann, 1942; Seneviratne, 1949).

The intrahepatic biliary system

The smallest unit of the bile drainage system is the bile capillary or bile canaliculus. These canaliculi lie in grooves between the parenchymal cells of the liver, (Popper, 1954). They communicate with each other to form a chicken-wire network within the liver cell plates (Elias 1949a). Although the existence of these canaliculi is not doubted, their structure appears to be uncertain. Popper (1954) suggested that they were adaptations or modifications of the membrane of the liver cells; Elias (1949a) believed that they were rigid structures which retained their integrity even after harsh treatment, such as maceration. Others have, however, shown that the canaliculi were fairly labile structures and that, under the influence of anoxia, they became a series of disconnected vacuoles (Hirt, Ansgorge and Markstahler, 1939; Grafflin and Bagley, 1952).

The bile canaliculi are thought to be joined to the cholangioles (or bile ductules) by the Canals of Hering. The existence of these channels between canaliculi and cholangioles has been shown most clearly in the rat by Hanzon (1952; 1958), but its existence in other animals (man, dog and cat) has been doubted by Andrews (1955; 1958). Andrews rejected the notion that the bile canaliculi were concerned with the excretion of bile and, therefore, had no need of the Canal of Hering. The more orthodox view that excreted bile has its origin in the bile canaliculi clearly demands some sort of connecting channel between the canaliculi and the cholangioles. It cannot be denied, however, that in some species such connecting channels have been extremely difficult to demonstrate, if at all.

The cholangioles are the smallest element of the biliary tree present in the portal tracts; they are lined by pavement-like epithelial cells different structurally and cytochemically from the liver cells (Maximow and Bloom, 1936; Popper, 1954).

These ductules develop from the liver cells embryologically and, in exaggerated fashion, during abnormal processes, especially in regeneration (McMahon

and Taunhauser, 1952; Elias 1955a).

The cholangioles join together to make longer tubules - the interlobular and sublobular bile ducts. These intrahepatic ducts then communicate with the extrahepatic ducts (Wakim, 1954). The blood supply of the bile ducts has been described in detail by Andrews, Maegraith and Wenyon (1949) and Maegraith (1955). These workers have shown that there is a good supply of arterial blood to the major system of the bile ducts. They described an elaborate plexus about the bile ducts (and gall bladder) in man, monkey, dog, cat, guinea pig and rat. Using hycar-latex injection methods, they showed that branches of both hepatic artery and portal veins ran freely to the plexus from the vessels of the portal tracts, and short vessels arose from the plexus and returned to the sinusoids directly.

The bile canaliculus has not a separate wall or limiting membrane of its own, but is, in fact, a tubular intercellular space formed by the apposition of corresponding grooves in the surface of the two adjoining cells according to Fawcett (1955) using the electron microscope. Thus, in the rat liver, each of the two cells constitutes one half of the

wall of the canaliculus. The portion of the cell surface which forms part of the wall of the canaliculus is studded with short processes, or microvilli, which project into the lumen of the bile capillary. These are similar to the cell processes of the striated border of intestinal cells, but are much shorter.

The lymphatic drainage of the liver

There is no reference to the first demonstration of the liver lymphatics although this is usually attributed to Saunders in 1809. Saunders' work was described by C.J. Watson during a discussion following a paper on liver lymphatics by Bollman in 1950. Saunders increased the pressure in the common bile duct of the dog and noted the lymphatics of liver became yellow. He also demonstrated a return of bile into the blood of the hepatic vein, and concluded that when there was increased pressure in the common bile duct, there was a regurgitation of bile both by way of the blood and by way of the lymph. He noted, further, that regurgitation of bile into lymph occurred earlier and to a greater extent than it did into the blood. Starling (1894) confirmed Saunders' observations and added some of his own observations. These, however,

are unacceptable because the mutilating operations performed (e.g. hepatectomy and ligation of the inferior vena cava) render the animals unrepresentative physiologically, of the intact animal. More precise investigations in trained, lymphatic-fistula dogs (Cain, Grindlay, Bollman, Flack and Mann, 1947) gave the average rate of flow for hepatic lymph as 2.26 ml/10 min. and for the thoracic duct lymph 4.6 ml/10 min. It was concluded from these observations that the liver probably contributed one quarter to one half of the total volume of lymph drained by the thoracic duct.

Ritchie, Grindlay and Bollman (1959) made observations of the hilar (periportal) lymphatics of the liver and in 100 dogs found two main drainage pathways (a) a main hilar system, draining predominantly the right lobes, and (b) an accessory hilar system, draining mainly the left lobes. These workers also showed that normally 80% of the lymph leaving the liver travels by the hilar route, and the remaining 20% by the hepatic venous lymphatic route.

The finer ramifications of the hepatic lymphatics are a matter of considerable interest. No lymph channels have ever been demonstrated within the liver lobule itself, i.e. in close association with

parenchymal cells. Mall (1901) believed that the lymphatics of the liver arose in the portal tracts and 'perilobular spaces' i.e. outside the liver lobule itself. He postulated, but did not demonstrate beyond douht, a direct communication between tissue fluid spaces (peri-sinusoidal spaces of Disse) and lymphatic vessels. Lee (1925) also failed to demonstrate lymphatics within the liver lobule. He described a rich plexus of lymphatic vessels in Glisson's sheath and liver capsule, which extended up to, but not within, the liver lobule. He observed many anastomoses between portal units and abundant communications with similar vessels in the walls of the hepatic veins.

Johnson and Mann (1950) observed intimate association of lymphatics with all components of the liver, especially the biliary system. Numerous lymph channels were seen completely surrounding the finer branches of each bile duct, and in the smaller bile ducts, lymph vessels were demonstrated just below the duct epithelium.

A reasonable view of the origin of liver lymph then, would be that the fluid in the perisinusoidal spaces of Disse passes to the tissue spaces in the

periphery of the portal tracts (Space of Mall) and there comes into association with lymph vessels. The presence of lymph channels closely related to the bile ducts may be regarded as an additional or alternative source of liver lymph.

The innervation of the liver

The liver, gall-bladder and biliary tract receive their supply from the sympathetic and parasympathetic systems and from the right phrenic nerve. Most of the sympathetic postganglionic fibres originate probably in the coeliac ganglia, some of these fibres may start in one of the small ganglia present at the hilum of the liver. The parasympathetic innervation is provided by both vagus nerves.

Within the liver, the nerves follow the branches of the blood vessels and bile ducts. The distribution within the liver is not precisely known. The portal tracts contain small non-myelinated fibres, sometimes intermixed within larger myelinated fibres. In the wall of the bile ducts a nerve fibre network extends close to the epithelium. Fine fibres have been demonstrated in the interlobular tissue spaces, but it is not known whether or not these fibres reach the liver cells themselves. The branches of the hepatic artery are

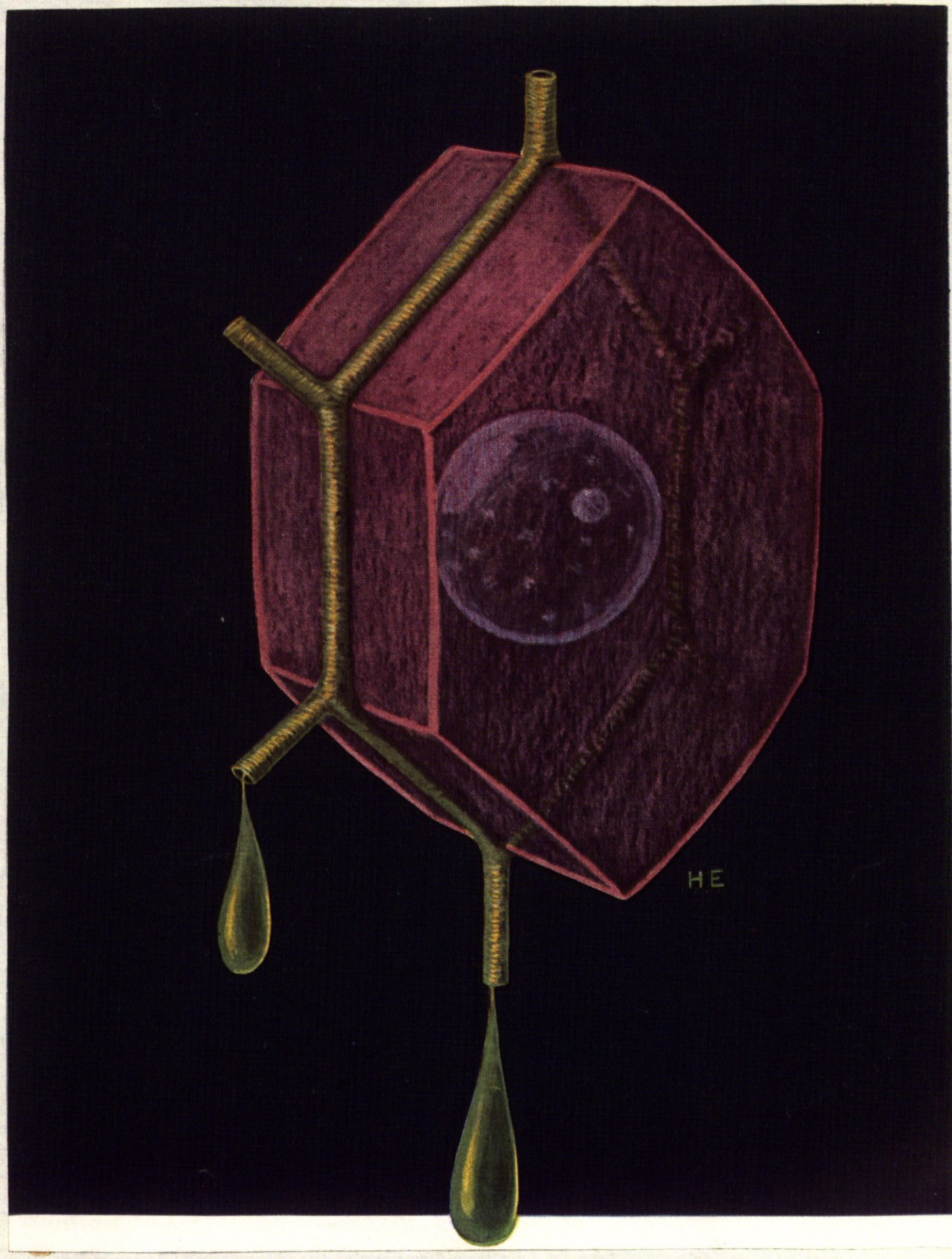
supplied entirely by sympathetic fibres, while the musculature of the bile ducts is innervated by both autonomic nerves.

III THE LIVER CELL

Figure 16.

A symbolic presentation of the hepatic cell.

(Taken from Research in the Service of Medicine, Volume 37, G.D. Searle and Company, Ltd.,)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The intimate arrangement of liver cells, their blood supply and the bile-draining apparatus has been dealt with in previous sections in the Introduction. The micro-anatomy of the liver was also discussed and it has been noted that despite the multitude of functions hepatic cells perform collectively, their individual appearance does not differ greatly from other cells of the body.

In this Chapter on the Liver Cell, the cell structure is detailed initially and following this two special features of cell function are discussed - one applicable to tissue cells in general and the other specific to liver cells. The first, being the problem of the cell and secretion and the second deals with the limited capacity of hepatic cells to eliminate substances into the bile. The ability of the liver to deal with the dye bromsulphthalein, when it is introduced into the circulation, depends to some extent on these features, as well as the peculiar internal anatomical arrangements of the liver.

1. THE DETAILED STRUCTURE OF THE LIVER CELL

Fawcett (1955) described the ordinary light microscopic appearance of the rat liver cells as follows :-

"The cells of rat liver are typically polygonal in shape. The nucleus is round, has a sharp outline and contains one or more prominent nucleoli and a few small clumps of chromatin situated close to the nuclear membrane.

The cytoplasm presents a mottled appearance, with coarse, angular masses of basophilic material alternating with pale or unstained areas. Basophilic bodies are known to be rich in ribonucleic acid, and the paler area of the cytoplasm can be shown to contain glycogen.

The mitochondria are numerous and appear as plump rods or slender filaments, depending on the physiological state of the animal and the location of the cell within the hepatic lobule.

The Golgi complex is commonly found at the periphery of the cell near a bile canaliculus,

but it may be juxta-nuclear.

The bile canaliculi appear as minute intercellular openings situated midway along the surfaces of adjacent cells.

Cells are arranged in plates, one cell thick, and their principal support seems to be a loose feltwork of reticular fibre which is interposed between the parenchymal cells and the cells lining the sinusoids".

Fawcett (1955) then described the appearance of these structures as seen by the electron microscope.

The nucleus is usually deformed to an oval shape. The karyoplasm is preserved in the form of granules of uniform size (150 - 200A^{o}) and moderate density. Granules are thinly dispersed in some areas whilst in others they are aggregated into ill-defined grey masses of irregular outline. The distribution of these darker areas within the nucleus corresponds fairly well with the pattern of the chromatin seen in stained sections with the light microscope, and it is possible that the component particles consist of desoxyribonucleo-protein.

A second kind of granule, less abundant and more osmiophilic than those just described, is found in small clusters of varying size (80 - 240 mμ) scattered through the karyoplasm.

The nucleolus is composed of a coarse strand (125 - 200 mμ) which branches and anastomoses to form a tight mesh-work of tangled skein. Higher magnification shows this strand to be made up of particles, densely packed granules 150 - 200A° in diameter. These granules bear a strong resemblance to the particles which are associated with the basophilic component of the cytoplasm, and are presumed to be ribonucleo-protein.

The nuclear membrane consists of 2 membranes of nearly equal thickness (about 90A°) pursuing a parallel course 100 - 200A° apart. Occasionally the two membranes appear to be continuous with each other at the margin of small openings or pores (300A°) through which these seems to be a free communication between cytoplasm and karyoplasm.

The Cytoplasmic Matrix and its inclusions :- The cytoplasm has a heterogenous appearance with dense mitochondria and irregularly shaped basophilic bodies sharply outlined against a less dense

background which has a fine granular character. Glycogen is quite uniformly dispersed throughout the cell.

Small vacuoles are frequently observed near bile canaliculi; some of these are no doubt related to the secretion of bile.

The Basophilic Substance (or ergastoplasm):- Later work using better methods has shown the older lamellar view to be inadequate. It is now seen that the basophilic component is a complex system of inter-communicating, membrane bounded tubules and vesicles. The term 'endoplasmic reticulum' has been applied to this organelle. In the liver cells, endoplasmic reticulum occurs in the form of tubules which pursue a meandering course through the cytoplasm, but in many places the tubules are expanded into broad flat vesicles.

The mitochondria are seen as plump cords or short filaments randomly orientated in the cytoplasm. Individual mitochondria are very commonly partially encircled by a strand of the endoplasmic reticulum (Piccardo, 1955). Each mitochondria is enclosed in a pair of membranes of which the outermost is smooth and continuous, while the inner one is thrown into

narrow folds which project inward like a series of baffle plates.

The Golgi complex is seen as an elaborate aggregation of parallel, double membranes, small vesicles and large vacuoles containing from one to several osmiophilic granules. This peculiar membrane-limited system resembles none of the forms commonly ascribed to the 'Golgi apparatus' of classical cytology, but there seems to be little doubt of its identity. It bears superficial resemblance to the parallel double membranes of basophilic bodies but they are closer together and can also be distinguished by the fact that they lack the small dense particles which are usually an identifying feature of the basophilic component of the cytoplasm.

2. THE CELL AND SECRETION.

Secretion is often defined as the production by the cells of substances necessary to the body and excretion as the elimination of useless substances, particularly the waste products of metabolism (Heilbrunn, 1943). When cellular activity is considered it is difficult to differentiate between these two processes.

From the point of view of cell physiology, there is no definite reason to distinguish between these processes wince very little is known of the actual functions of the cells in either process. Hober (1942, 1946) refers to the mere transport of substances through the cells by the term secretion, but at the same time points out that in other cases even more complicated changes of substances may form part of the secretion process. In the present work the word 'secretion' has been used in Hober's sense. Only the form of secretion affecting the mere transport of substances through the liver cells has been subjected to investigation. Accepting that with secretion substances pass through the cells, there are two factors affecting the rate of transport (i) the cell permeability for the substance under examination and (ii) the 'driving force' acting on that substance (Brooks and Brooks, 1941).

Cell permeability

A body is said to be permeable to a substance if it allows the passage of the substance through itself. Permeability has been defined as the rate of movement of a substance through the permeable layer under a given driving force (Brooks and Brooks, 1941).

The permeability of the cell is considered to be dependent on certain parts of the cell being less permeable than the bulk of the protoplasm. These parts are assumed to be the interfaces of the cells (i.e. superficial or peripheral, nuclear and vacuolar). The permeability that regulates the passage of substances between the cell and its surroundings is believed to be localized to the 'membrane' that surrounds the cell as the most superficial layer of the cytoplasm. The membrane probably consists of layers of lipoid and protein molecules.

The permeability of a medium by a substance is dependent on the nature of the substance and the properties of the medium. The capacity of a large number of substances to permeate cells has been investigated, and these investigations to a great extent form the background of theories concerning the structure of the cell membrane. A definite connection between the lipoid solubility and the penetration capacity of substances has been observed, as well as a distinct relation between molecular size and penetration capacity (Overton, 1899; Collander and Barlund, 1933). These two fundamental observations have led to the 'lipoid sieve' theory,

according to which the membrane consists of lipoids through which substances can pass owing to their lipoid solubility. Those substances insoluble in lipoids may pass through pores in that membrane with the rate being dependent upon the molecular weight of the substance.

Although little is known regarding cell permeability and the manner in which substances can penetrate cells, it is thought that fundamental physical processes play a part in this connection. Collander and Barlund (1933) found that certain non-electrolytes follow Ficks law, i.e. penetration by pure diffusion, but in many cases it is not as simple as that. According to De Robertis et al (1948) four mechanisms are known to regulate the passage of substances to and from cells, which may explain the selective permeability in different cells. The four mechanisms are lipoid solubility, penetration through pores with or without electric charge and iron exchange. However, the same authors emphasise that several other factors undoubtedly play a part, especially to account for the passage of larger molecules. Danielli (1950) divides the mechanism into five groups, (i) molecules pass

together with an abundance of water through the pores in the membranes (filtration), (ii) molecules pass through by thermal diffusion through the water in pores in the membranes (iii) molecules pass from the aqueous phase to the non-aqueous phase of the membrane, the principal resistance being found at the border surfaces between the two phases, (iv) molecules pass in the same way between the two phases, but the chief resistance occurs in the inner part of the membrane, (v) all other forms of passage through the cellular membrane including forms with a secretory activity in the cells.

The permeability of the cell is not constant and is subject to variations in different physiological conditions in the body. It is dependent on the internal invironment of the cell i.e. physical and chemical conditions, the presence of certain salts and fat solvents, irradiations of different kinds. Heilbrunn (1943) showed that injury to the cell or its death, cause marked changes in permeability. Different injurious agents increase the permeability of the cell to water and other substances, and the selective permeability of the cell is diminished or lost and it lets through all sub-

stances.

Increased permeability in cellular membranes is regarded, at least partly, as the reason for the higher content of dyes in injured cells at vital staining as described in liver, kidney and muscle by Oliver, Bloom and MacDowell, (1941), Williams (1947, 1948), Williams and Frantz (1948) among others. A similar change in the membrane of the nucleus is also the reason for its being stained in degenerated or injured cells at vital staining (Chambers, 1922, Ludford, 1933).

The 'driving force'

The other factor, which together with the cellular permeability governs the passage of substances through the cells, is the 'driving force'. This may consist of concentration gradients, hydrostatic pressure gradients, electric potential gradients or surface gradients (Teorell, 1949). The 'driving force' may either be independent of the cell through which the passage of substances occurs or be produced by processes within this cell, requiring energy.

Chambers (1935) found that basic dye stuffs occur in renal tubular cells, irrespective of great variations in the temperature which affected the

metabolic rate, apparently by passive infiltration of the dye. He propounded that substances can pass in or out of the cells without requiring any energy from the cells, the 'driving force' being, for instance, a concentration gradient. Hober (1946), Dean (1947) among many others have shown that the passage of substances through cells, even against the concentration gradient, may be accounted for by processes not requiring energy from the cell.

In many cases of the transport of a substance through cells, the process cannot be attributed only to diffusion, osmosis or other specific physical processes when only the cell and its surroundings are taken into account. It may prove dependent on the metabolic activity in the cell and it may be assumed that 'driving forces' are produced by specific processes in the cell requiring energy. It is characteristic of this form of transport of substances that it takes place in a direction against the concentration gradient. Water can be transported in a direction against hydrostatic and osmotic pressure. Hober (1942, 1946) called these processes requiring energy from the cell 'active transfer', while the transport of substances attributable to

physical reactions irrespective of cellular activity was called 'passive penetration'. The capacity to transfer substances actively is more pronounced in certain cells, particularly in gland cells and cells with phagocytic or strong metabolic functions (Hober, 1946). Lison, (1938), Hober, (1946) and Danielli (1950, 1951), as well as other workers, mean by secretion in its actual sense, an active transfer, utilizing energy from the cells.

The active transfer of substances through the cells seems to be dependent on a specific affinity between the transported substances and some part(s) of the cells. The properties of the substance of significance in this respect may vary from one case to another. Unspecific properties, such as molecular size and lipoid solubility which may be decisive in passive penetration, are of small significance for the rate of active transfer, unless the substance is colloid and inaccessible to the cells. The rate of transport by specific cellular processes is of an entirely different order of magnitude than the transport of substances dependent on these unspecific factors (Shannon, 1939).

Dye stuffs have been used a great deal for

studying the process of active transfer. The acid dyes are most suitable in this respect. It is characteristic of many of them that while many cells fail to show any colour after long exposure to strong solutions of these dyes (in spite of being readily diffusible and non-toxic), other kinds of cells take up the dyes rapidly and copiously; the molecular structure of the dyes being essentially significant. This uptake has been referred to as representing an active transfer brought about by living cells (Hober, 1946). The connection between the molecular configuration of dye stuffs and their active transfer has been discussed by Hober (1940, 1942). The processes underlying the histological staining of cells in so-called vital staining, as usually carried out, are of an entirely different nature.

The source of energy required for active transfer of substances through cells is derived from the metabolic processes occurring in the cells. If these processes are stopped, the movement of the transferred substance is inhibited. Many experiments have been carried out to investigate the dependence of the transport of substances on energy

from the cells and to find out more precisely where this energy originates. In some of these experiments it has been possible to show that the energy originates from the breakdown of carbohydrates, because the blocking of the carbohydrate enzyme systems by different agents affects the concentration capacity of dyes reversibly in the liver and kidney (Ferrari and Hober, 1933, Koll-Schroder, 1934, Selkurt, 1951). Many of the energy-emitting processes are aerobic processes and anoxia considerable affects them (Chambers, Beck and Belkin, 1935). The transport of substances may also be markedly affected by the temperature of the process (Chambers and Kempton, 1933; Danielli and Dawson, 1935).

At least two seats of activity may possibly be distinguished by secretion, i.e. the surface of the cell and the interior of the cell, but very little is known concerning the mechanism (Danielli, 1951). However, a few facts have been presented and many theories laid down postulating the mechanism of active transfer.

In different processes with an active transfer of substances through the cells, it has been possible to find out that the amount of substances transported

per unit time through the cell is a non-linear function of the concentration of that substance in the environment. When the concentration rises, the transport mechanism tends to become saturated and the rate of transfer increases more slowly than the concentration in the environment. This was established in the intestinal absorption of glucose by Auchinachie, Macleod and Magee (1930) among others, and in renal tubular secretion of phenol red and many other substances by Shannon (1935), Pitts, (1938), Smith, Goldring and Chasis (1938), Forster (1940) and others. Further, Smith and Ranges (1938) demonstrated that when several substances are secreted simultaneously through the cells in the renal tubuli, each one of them cannot attain the same concentration in the urine as when secreted separately at a higher concentration of the substances in the blood. From this they concluded that these substances were actively transferred by means of a particular mechanism with a limited capacity to a substance per time unit. The cellular component of the mechanism may be common to the transfer of more than one substance, and when several of these substances are transported simultaneously, they enter

into quantitative competition for it. This cellular component seems, to judge from the different experiments, to be more or less a stable feature in the organization of the cell which does not arise immediately on presentation to the cell of the substance that is to be transported. However, when this concentration increases, the rate of transfer is in addition determined by the limitation of the cellular transport mechanism (Shannon, 1939).

Some authors have stressed the part played by the cell membrane in active transfer, regarding the membrane as an actively working part of the cell (Hober, 1947). The exact mechanism for the active work in the membrane has been suggested as being a chemical organisation in the cell membrane where different enzymes are localised to the inside and outside of the membrane. In this way, the substances might be transformed on one side so as to able to pass through the membrane, and then again be transformed into another form on the other side. Such an enzyme system may be regarded as a 'membrane carrier'. This has been particularly studied in connection with the transport of glucose (Hober, 1946, Rosenberg and Wilbrandt, 1952). It was pointed out

by Osterhour, (1952) that a similar mechanism existed for accumulating substances in the cells localised to the cell membrane. Accumulated substances are dissociated in ions which cannot permeate the cell membrane. It may then be assumed that for passage through the membrane, the ions combine with organic carrier molecules, a combination to which the membrane is permeable. These carrier molecules should be formed through cellular activity on the outside of the membrane. On the inside, this combination should again break up and the free ion be trapped there, the carrier molecule being destroyed, thus eliminating any possibility of a similar transport in the opposite direction. In both these mechanisms in the cell membrane, the driving force is the concentration gradient of the particular substance appearing near the membrane. This substance is not accumulated, as it disappears after passage through the membrane by transformation to another form. The accumulation that takes place is due to the fact that the substance, in the form it has on either side of the membrane, cannot penetrate the membrane.

Other mechanisms have been suggested for the active transfer of substances that might be localised

to the cell surface as well as inside the cytoplasm. They are based on the contractility or capacity of folding and unfolding attributed to fibre molecules of a protein nature in the cells. Through such contractions, the size of the pores in the plasma membrane might be varied, possibly so as to produce a pump mechanism (Schmitt 1947, Monne 1948). The contraction waves in these fibres in the cytoplasm might even cause streams of fluid there, thus producing a transport of substances, or substances might be transported by a temporary binding to the parts of the protein molecules changed under the cobtraction, thus joining the contraction wave. The problems of folding and unfolding of protein molecules and the importance of that phenomenon for fundamental cell processes, among which the performance of osmotic work or secretion has been reviewed by Goldacre (1952) and Seifritz (1952). Danielli (1952) suggested that the contractile proteins may be phosphatases.

The part played by vacuoles or granules in the transport of substances in secretory cells is uncertain. Ludford (1931) showed that only slowly diffusing acid dyes are accumulated as granules in the liver cells, not the readily diffusing acid dyes.

He considered this to represent the 'normal path of excretion'. Among the cellular structures appearing in all cells, the Golgi apparatus in particular has been directly connected with secretion (De Robertis, Nowinski and Saez, 1948, Bourne, 1951). Some dyes e.g. the slowly diffusing trypan blue) are often seen as granules or vacuoles in Golgi apparatus in liver cells (Ludford 1928, Pfuhl and Dienstbach, 1938). In several gland cells, a morphologic connection has been noted between the secretory granules and the Golgi apparatus, as well as a variation in the appearance of the latter is different phases of the secretion (Cramer and Ludford, 1926, Bowen, 1926, 1929). Deane (1944) found no connection between the activity in the Golgi apparatus and different stages in the bile secretion or secretion of trypan blue. The direct connection of the Golgi apparatus with secretion seems only to have been investigated in such forms which involve an accumulation of substances in vacuoles or granules in the cytoplasm.

3. THE LIMITED CAPACITY OF LIVER CELLS TO ELIMINATE SUBSTANCES IN THE BILE

Several authors have reported experiments which favour the assumption that the liver cells have

a limited capacity to concentrate and eliminate substances in the bile, and that when more than one substance is secreted simultaneously, they must compete for the secretion mechanism.

Brauer and Pessotti (1950) using a continuous infusion of bromsulphthalein, found the concentration of dye in the bile only rises to a certain limit value when the concentration in the blood is increased. Lewis (1950) showed that the amount of bromsulphthalein eliminated from the blood in rising concentrations only reaches a certain limit.

Dragstedt and Mills (1936) reported that bromsulphthalein was retained in the blood for a longer time than normal if bilirubin was administered before bromsulphthalein. When bilirubin and bromsulphthalein were injected simultaneously, bromsulphthalein was found to be excreted as effectively as if it had been given alone, but less bilirubin was obtained in the bile than when administered alone (Cantarow, Wirts, Snape and Miller, 1948, Wirts, 1949). Snapp, Gutmann, Li and Ivy (1947) reported that the clearance of Rose Bengal was decreased by bilirubin. Rose Bengal and bromsulphthalein affect each others elimination in the bile, bromsulphthalein having a

marked inhibitory effect on the removal of Rose Bengal from the circulation, whilst Rose Bengal has only a minor inhibitory influence on the disappearance of bromsulphthalein from the blood (Cohen, Giansiracusa, Althausen, 1953). The elimination of Rose Bengal and bromsulphthalein may be temporarily blocked by dehydrocholic acid (Mendeloff, 1949 a,b). Dehydrocholate also reduces the amount of exogenous bilirubin traceable in the bile, although the effect appears to depend upon the relative doses of dehydrocholate and bilirubin (Berman, Snapp and Ivy, 1941). The amount of bromsulphthalein eliminated per time unit in the bile decreased temporarily at maximal choleresis produced by dehydrocholate (Cantarow, Wirts, Snape and Miller, 1948). Benemid also decreases the excretion of bromsulphthalein, particularly when given before the dye (Goetzee, Richards and Tindall, 1958). India ink was also considered to interfere with the excretion of bromsulphthalein, but Zilversmit, Shore and Gantt (1954) showed that it was due to the toxic action on the liver cells of one or more of the components of the dispersing medium present in India ink on the liver cells.

This limiting capacity for the transport of a substance through the liver is more pronounced in regard to the elimination of substances into the bile than the uptake of the substance from the blood. A dual mechanism for bile secretion has been postulated in which the uptake of a substance from the blood is more rapid than the elimination in the bile, with the result that the substances that are to be eliminated are stored temporarily in the liver cells, or perhaps in the Kupffer cells (Wirts and Cantarow, 1942, Cantarow, Wirts, Snape and Miller, 1948, Wirts 1949, Brauer and Pessotti, 1950, Wirts, Cantarow, Snape and Delserone, 1951).

According to Cantarow et al, their experiments indicate that such a storing takes place in the liver even when the substances administered are calculated on being diluted out into the blood and all the extracellular fluid. Brauer and Pessotti (1950) found that only the elimination of the substances into the bile reaches a maximal value at a rising plasma concentration, the uptake from the blood being increased throughout. This was explained partly by the extra-hepatic storage of the dye, but must also be due to a storing of the substance in the liver

cells, as well as, possibly its destruction. Hanzon (1952) using the more direct method of fluorescence microscopy to study liver cell secretion was able to confirm the dual mechanism assumed for indirect reasons by Cantarow et al, Brauer and Pessotti. As a result of his study, Hanzon considered the slower elimination of substances into the bile compared with the rapid uptake from the blood, could well be due to the more limited capacity of the mechanisms in the smaller area of cell surface facing the bile capillary as compared with the larger cell area facing the circulating blood.

Lewis (1950) found a maximal secretory capacity for the disappearance of bromsulphthalein from the blood in the rabbit, and Hanzon (1952) found that bilirubin has a similar value when calculated in molecules. It is highly probable that the limited concentration of bilirubin in bile is due to an incapacity of the cells to handle more than a maximal amount of the substance per unit of time i.e. a 'secretory ceiling' exists in the liver cells for bilirubin. A 'secretory ceiling' for alkaline phosphatase in the liver has been reported by Le Veen, Talbot, Restuccia and Barbato (1950) so that only a limited quantity could

be eliminated per unit time. The existence of such a ceiling has also been demonstrated by Brauer and Pessotti (1950) with regard to bromsulphthalein. The occurrence of a secretory ceiling in the liver cells would suggest that the secretion (i.e. active transfer) is performed by some kind of carrier mechanism.

IV THE BILE

1. THE FORMATION OF BILE

Most workers are agreed that bile is produced by the parenchymal cells of the liver with the exception of Andrews (1955) who suggested the bile duct cells. Cramer and Ludford (1926) observed that the bile constituents appeared as a secretion within the Golgi apparatus which, in the process of secretion, first enlarged and then fragmented. The fragments were dispersed through the cytoplasm and reached the periphery where they passed their contents into the intercellular bile capillaries. Although there is almost universal acceptance of the role of the liver cell as the site of bile production, there is no generally agreed view on the processes involved. These processes are not completely understood at present.

It is known that bile contains substances which also appear in the plasma, the mechanisms for these substances to be transferred from the plasma to bile are the subject of a lot of research with as yet an incomplete answer. Whether or not the method of transfer involves filtration alone, or active secretion alone or a combination of these also remains unknown.

The experimental findings can be interpreted in two ways. Bizard and Vanlerenberghe (1956) have, for example, suggested that both filtration and active secretion are involved in bile production, whereas Brauer (1959) believes that active secretion is the only process concerned.

It is helpful to elaborate these two different views since it also reveals the difficulties of interpreting any of the bile formation data. Many independent workers share the views of Bizard and Vanlerenberghe (1956). These workers recognised that in the excretion of foreign substances, the liver behaved in two different ways. Some substances (e.g. dyes) were concentrated many times in the bile; other substances (e.g. sugars) seemed to pass through the liver without any great change in concentration. Heywood and Hober (1937), using frog liver, showed in a striking way the differences between these two classes of substances. They perfused the frog liver with Ringer solution to which a dye erythrocyanin (molecular weight 500) and a sugar such as xylose, lactose or inulin had been added. In all cases, the dye excreted in the bile was in a concentration many times that of the perfusate, whilst the sugar passed

into the bile without any change in concentration. Even inulin (molecular weight 5,100) passed through the liver without change of concentration. These workers, therefore, concluded that with regard to the sugars xylose, lactose and inulin, the liver behaved as a passive filter of which the pore size was limited, but sufficiently big for the large molecule of inulin to pass through. The dye (erythrocyanin) they regarded as being actively secreted.

These results were later confirmed by Heywood (1946) in the living animal. Heywood measured the inulin concentration in the bile and blood after intra-muscular injection into the living toad-fish, which has an aglomerular kidney and does not excrete inulin. In all his experiments he found that the concentration of inulin in the bile was less than the concentration of inulin in the plasma. The ratio of bile inulin/plasma inulin was always less than 1.0 (mean 0.56). Heywood regarded these experiments as confirming that inulin was passed into bile by a mechanism of passive filtration. Bizard and Vanlerenberghe (1956)

suggested that there were two possible explanations for the low bile/plasma inulin ratios :- either (a) the inulin was filtered by one pathway and was then diluted with water reaching the bile by another route or (b) a portion of the inulin was retained in the organism - in this the 'pore' diameters of the filter would have been very near the actual molecular size of inulin.

This type of result has also been obtained in mammals. Cook, Lawler, Calvin and Green (1952) gave continuous infusions of inulin to dogs with an acute biliary fistula and found the bile/plasma inulin ratio to be 0.47. Cook et al also made measurements of the rate of biliary excretion, the bile/plasma concentration ratios, and biliary clearances of a variety of substances including Na^+, K^+, Cl^-, glucose, inulin, cholesterol, creatinine, bromsulphthalein, para-amino hippurate, bilirubin. Their findings were accepted as evidence that two principle mechanisms existed to explain the appearance and amount of any substance in bile. These two mechanisms were filtration, and active secretion by the liver cell.

Of the substances studied by Cook et al (1952)

water, Na^+, K^+, Cl^-, glucose, inulin, cholesterol and creatinine were regarded as passing into bile by filtration, their bile/plasma concentrations were near unity (in fact ranged from 0.5 to 2.0). Other substances (bilirubin, bromsulphthalein, para-amino hippurate, penicillin) were excreted into the bile at a concentration many times that present in the plasma. It was therefore concluded that these substances were eliminated from the liver by a process of active secretion. The view of those workers who accept both filtration and active secretion as processes necessary for bile formation may be summarised as follows :-

Water, electrolytes, and certain crystalloids are excreted by the liver into the bile by a process of filtration or diffusion, whilst biliruhin, dyes and certain antibiotics are actively secreted. There is some possibility of reabsorption of water and certain ions.

However, even Bizard and Vanlerenberghe were not entirely satisfied by this explanation. They conceded that filtration plus reabsorption of water was not sufficient to explain why some substances

were eliminated at high concentrations and others not, and they noted the relative indeoendence of bile flow and blood flow in the liver - a circumstance which could only be accepted by a hypothesis of active secretion of bile.

Brauer's view that the formation of bile is the result of processes of active secretion has been based very largely on perfusion experiments using isolated rats livers (Brauer, Leong and Holloway, 1954; Holloway and Brauer, 1954; Leong, Holloway and Brauer, 1955). These workers have used the isolated rat liver preparations to study the inter-relationships of temperature, perfusion pressure and perfusate flow on the one hand, and bile flow and bile secretion pressures on the other. They allowed bile to flow in a vertical catheter and noted that the pressure indicated by this catheter when bile flow ceased was much greater than the perfusion pressure. They observed that bile production ceased at a temperature of approximately 24°C and was maximal between 38 - 40°C.

Brauer and his colleagues concluded from these, and similar experiments, that bile is formed as a secretion analogous to other digestive glands,

and that bile formation requires the application of metabolic energy for the performance of osmotic work. The importance of this conclusion lies in the fact that active secretion is not only demanded for substances which appear in bile in high concentration, but also for those substances (mainly electrolytes) whose concentration in bile and plasma is almost identical. Brauer (1959) divided bile constituents into three classes :-

Class A :- Na^+, K^+, Cl^-, glucose i.e. those substances in which the blood/bile ratio is 1:1.

Class B :- Taurocholates, glycocholates, bile salts, bilirubin, diglucuronide and other bile pigments, bromsulphthalein, fluorescein, and Rose Bengal i.e. substances whose blood/bile ratio is in the range 1:10 to 1:1,000.

Class C :- Alkaline phosphatase, inulin, sucrose phosphates and possibly cholesterol, phospholipids, mucoprotein, i.e, substances whose blood/bile ratio is 1:1.

Class A and B substances account for water and 95% of dry bile. Brauer accepted that Class B substances are actively secreted into bile and he regarded bromsulphthalein as a typical example of this type of substance. He reviewed the evidence available which pointed to the active secretion of bromsulphthalein but this will be considered later on in the chapter dealing with bromsulphthalein and the liver.

Brauer's (1959) views on the Class A are as follows :-

"These substances, almost by definition, are transferred to bile in a group and their transfer bears no necessary relation to the rate of transfer of Class B compounds secretion. The fact that Class A substances as a group appear in bile in concentrations closely reflecting those of the plasma has suggested to some that a filtration-reabsorption process is the basis of bile colume control. This is, however, incompatible with the experimental facts.

Thus filtration can be ruled out because bile flow continues up to limiting pressures which, in the isolated rat liver, are always greater than blood pressure. If, furthermore, one takes into account

the effective colloid osmotic pressure of the plasma proteins (all but one or two minor ones of which are absent from bile, a pressure differential of such magnitude results that any filtration process can be ruled out. Even a second order contribution of blood pressures to bile flow rates can be ruled out, since bile flow rates are independent of blood flow or blood pressure whenever the oxygen supply to the tissue is not a limiting factor.

Active reabsorption can also be ruled out as a factor in bile volume regulation. Under constant blood flow conditions cooling of the liver results in a sharp fall in bile flow rate not a rise, as would be expected from any mechanism dominated by reabsorption. Thus it would appear that, in essence, bile formation involves the active transfer of water and possibly other Class A substances from blood to bile.

Further light on blood-bile relations is shed by study of other Class A components as the monovalent ions Na^+, K^+, and Cl^-. The concentration of all these in bile rigorously parallels their concentration in blood plasma, regardless of how much deviation from normal blood levels is seen. This equality of plasma

(in the rat) and bile electrolyte concentrations and osmotic pressures is preserved even when the plasma osmolarity is doubled by the addition of sodium chloride. Bile flow rate is however drastically reduced by such a procedure, indicating once again that a cellular phase rather than extracellular equilibrium govers bile flow rate. The location of this cellular phase is suggested in this case by the watery vacuolation in the hepatic polygonal cells. The dominant role of the hepatic cells in bile secretion is further confirmed by the observation that in any forms of toxic hepatitis, where bile suppression is among the earliest physiological manifestations, early histopathological changes are confined to these hepatic cells. Thus, transfer of water to bile involves an active, energy consuming process, and is likely to be located at the level of the hepatic cells.

Once in the biliary tree, bile is not completely isolated from the blood stream. Thus, a new transfer of fluid out of the biliary tree could be demonstrated at elevated bile pressures. The rate of such back flow is dependent on bile pressure; it appears to be nil or very slow, relative to bile

secretion, at pressures up to about 12 cm. H_2O, and increases very rapidly with further increases in bile pressure until back flow balances secretion rate.

Bile composition appears to be independent of bile pressures below 10 to 12 cm. (of bile) and varies at higher pressures in a manner suggesting interference with the secretory apparatus rather than concentration of bile by selective back filtration.

Exchange of electrolytes can occur between bile in the biliary tree and blood plasma. This follows in particular from studies of bile K^+ concentration. After radioactive K^{42} injection into the blood stream, the specific activity of the large intrahepatic K^+ pool rises somewhat gradually while the bile potassium specific activity has reached the values prevailing in the plasma within about one minute. Bile potassium is thus derived from the blood and not from the liver cells. The rate of K^{42} appearance, furthermore, suggests that this ion entered the biliary tree pretty well downstream rather than in the canicular headwaters. Similar results have been found for Na^+ and Cl^-. Hence the suggestion that bile is secreted by the hepatic cells in passage down the biliary tree is afforded an opportunity of

reaching electrolyte equilibrium with the blood plasma. It is tempting to assign this process to the small bile ducts and ductules with their investing vascular plexus".

This slightly confusing review of his own work by Brauer is typical of the confusion surrounding the interpretation of experimental data on bile formation. The experimental data of Brauer leads one to the conclusion that there is good evidence for the belief that the water of bile is actively secreted but the remainder is inconclusive. The evidence for the active secretion of ions is inconclusive, the data of Holloway and Brauer (1954) and Leong et al (1955) can be interpreted only as suggesting active secretion of ions or a mixture of filtration plus active secretion.

It seems probably that a reasonable view of bile formation is one which accepts the evidence for active secretion by the liver of all the constituents of bile, except the electrolytes. These may be secreted but, equally, they may be passively filtered from the blood to the bile. If one accepts this view then the work of Tanturi and Ivy (1938b) is understandable. They found that, in the dog and monkey,

stimulation of the peripheral end of a cut vagus nerve resulted in an increased flow of bile and that this increase was not accompanied by any change of blood pressure. They did not estimate the constituents of bile before and after vagal stimulation but at least one can accept that bile water was increased, and that the increase was due to increased secretory activity by the liver cells following stimulation of the vagus.

2. FACTORS INFLUENCING THE PRODUCTION OF BILE

The relationship between blood flow through the liver and bile formation has been examined in detail by Tanturi and Ivy (1938a, 1938b). Using anaesthetised dogs with acute biliary fistulae, these workers observed the effect of section and stimulation of splanchnic nerves on bile formation. Section of the splanchnic nerves produced (in 9 experiments out of 11) an increase in the secretion of bile (mean 66% increase) which lasted for 5 - 8 hours, and no correlation was noted between the drop in arterial blood pressures and the increase in choleresis. Stimulation of the peripheral end of the cut splanchnic nerves produced ('in general') a reduction in bile flow,

and this phenomenon occurred even in the presence of a choleresis produced by bile salts. Tanturi and Ivy concluded that the changes observed in bile flow were due to vasomotor action and not to a specific action on the liver cells themselves. They also made several other observations :- (i) constriction of the hepatic veins caused a reduction of the bile flow, (ii) complete obstruction of the hepatic vein resulted in a complete stoppage of bile flow, (iii) complete occlusion of the portal vein resulted in a reduction of bile flow, (iv) complete occlusion of the hepatic artery caused a marked increase of bile flow (25 - 200% of control) and this effect lasted 2 - 5 hours. Tanturi and Ivy believed that an increase in liver blood flow favoured an increase in bile flow, provided that at the same time there is no large rise in blood pressure which would mechanically compress the bile canaliculi. Their work is also interesting in that it showed that bile could be still produced even at very low blood pressures (20 - 40 mm. Hg.). This fact would appear to minimise the importance of a filtration process in the production of bile.

Brauer et al (1954) using isolated rat livers

showed that bile secretion depended more on flow rate than on pressure. They observed that if the perfusion pressure fell below 10 cc/min., the bile flow fell off rapidly. On the other hand above a flow of 33 cc/min., bile flow changed very little. This is in general agreement with an earlier finding by Ivy (1930) in the dog. Ivy reported that when blood was diverted from the portal venous system into the vena cava (Eck fistula) bile production fell by half.

The dependence of bile formation on oxygen supply has been reported by various workers. As noted above, Tanturi and Ivy (1938a) reported an increase in bile formation after ligation of the hepatic artery, but Grer and Geller (1948) showed that when the thoracic aorta was obstructed, the production of urine ceased at once, whereas bile was produced for 20 - 25 minutes thereafter, although at a decreased rate (25 - 80% less than the control). When the obstruction to the aorta was removed, bile was formed again at normal control rates. In animals inhaling a mixture of gases which contained 3 - 6% oxygen, bile flow was found to be less than normal (Chardon, Neverre and Jeannole, 1949).

It seems probably that bile formation depends more on the oxygen saturation of portal venous blood than on the oxygen brought by the blood in the hepatic artery, but there does not appear to be a ready explanation for the increase in bile flow after ligation of the hepatic artery.

The volume of bile produced also depends on the quantity of certain substances presented to the liver by the blood flowing through it. It has long been known, for instance, that bile salts (e.g. taurocholate) given by mouth or intravenously, will cause a great increase in bile flow (Whipple, 1922). The bile so produced contains more bile salt but less bile pigment than normal. Glucose has the effect of reducing the bile flow considerably, the water of bile being notably reduced. The substances which cause an increase in bile flow are called choleretics and the chief of these are the bile salts. The nature and function of the bile salts, with regard to bile formation, will be discussed in the next section.

The amount of bile produced per day in the conscious dog with a chronic biliary fistula has been measured by Kocur and Ivy (1938). They found that

when bile was returned to the duodenum and the dog was fed its normal diet, the amount of bile produced was 24 - 27 ml/Kg/day. If no bile was returned, the production of bile fell to 13 - 18 ml/Kg/day. A further fall in bile production was noted when the animal was given neither food or bile for seven days. The production of bile under normal circumstances (or when bile is returned to the duodenum of the non-fasting animal) seems to depend both on a substance in bile which is reabsorbed from the duodenum, and a substance in the diet. Whipple (1922) has indicated the relationship between these two substances, namely, that bile salts produce an increase in bile flow and food protein (especially meat protein) causes an increase in bile salt production.

In the fasting, anaesthetised dog with an acute biliary fistula, one would expect the amount of bile produced per day to be in the range 6 - 10 ml/Kg/day found by Kocour and Ivy (1938), for fasting conscious dogs with no return of bile. Cook, Beach, Bianchi, Hambourger and Green (1950) were able to confirm this and found that in 38 dogs the hourly output of bile was 0.26 ± 0.24 ml/Kg/body weight. This

is equivalent to 6.24 ± 0.58 ml/Kg/day.

The amount of bile eliminated by the liver of a normal human adult is given as 500 - 1,000 ml. per 24 hours by Wakim (1954). The daily output of bile in a female patient after the operations of cholecystectomy and choledochotomy was measured by Koster, Shapiro and Lerner (1936). They observed their patient for 16 days, and over that time the mean rate of bile production was 490 ml/24 hours (range 420 - 540). They also noted a difference between the sleeping and waking rate of bile production. During sleep, production was at the rate of 15.9 ml/hour, and during the waking hours it was 23.4 ml/hour.

3. CERTAIN CONSTITUENTS OF BILE.

The constituents considered here are bile pigments, bile acids and salts.

Bile Pigments.

Mann. Bollman and Magath (1924) showed beyond doubt that the reticulo-endothelial system, and not the liver alone, was the manufacturing centre for bilirubin, the main pigment in bile. Bile pigments are produced from haemoglobin and myohaemoglobin. Heamoglobin

is broken down to haematin and then to bilirubin. Bilirubin is the globin-free, iron-free fraction of haemoglobin, and is excreted by the liver through the biliary system into the gastro-intestinal tract (Wakim 1954). Since bilirubin can be manufactured anywhere in the reticulo-endothelial system of the body but is only excreted by the liver, bilirubin must exist in the plasma in some form. Watson (1942) has shown that bilirubin in the plasma is bound to the albumin fraction of plasma protein and Martin (1948) has suggested that the binding is in the ratio of 2 molecules of bilirubin to 1 molecule of albumin at pH 7.4. In normal conditions, in man, the plasma concentration of bilirubin is between 0.5 and 1.0 mgm/ 100 ml., with an upper limit 1.4 mg/100ml. as measured by various modifications of the Van den Bergh test, (McNee, 1922; Ducci and Watson, 1945; and Zieve, Hill, Hanson, Falcon and Watson, 1951).

In abnormal conditions in man (and the dog) two types of bilirubin can be demonstrated in the plasma. One is a water-soluble pigment which gives the direct Van den Bergh test and is characteristically found in obstructive jaundice. The other is a chloro-form-soluble pigment giving the indirect Van den Bergh

reaction and is characteristic of haemolytic jaundice and normal human serum (Cole, Lathe and Billing, 1954; Bollman and Mendez, 1955).

The difference between these two types of bilirubin has been made clear by the work of Billing and Lathe (1956) and Talafant (1956). They showed that the water-soluble, direct reacting pigment was bilirubin diglucuronide and the chloroform-soluble indirect pigment was simply bilirubin. They showed that the excretion of bilirubin by the liver involved its conversion into the diglucuronide. This was confirmed by Lathe and Walker (1958) who were able to demonstrate the synthesis of the glucuronide in vitro with animal and human liver. Lathe and Walker showed that the bilirubin conjugating system was present in cell particles (mitochondria) and they had some evidence to suggest that the livers of human, newborn infants were deficient in the enzyme required for the synthesis of bilirubin diglucuronide.

The Bile Salts and Bile Acids

Wakim (1954) stated "the cells of the liver, are as far as present knowledge indicates, wholly responsible for the production of bile salts and bile acids". The work of Bollman and Mann (1936)

indicated also that the liver is not only the site of formation of bile salts, but is also the site of their destruction and excretion. They observed that after removal of the liver, injected bile salts were quantitatively eliminated through the kidney.

All bile acids normally occur in bile as conjugates of either taurine or glycine. In the rabbit, conjugation is mainly with glycine; and in the rat conjugation is almost exclusively with taurine (Norman, 1954); in man, both conjugates occur in varying proportions. In man, there are mainly three bile acids present, i.e. Cholic, desoxycholic and chenodesoxycholic acids. They occur in varying proportions in different individuals and the proportions vary with time in the same individual (Bergstrom, 1958).

Much of the earlier work on bile acids (and salts) suffered from an important defect. Only one bile acid was measured (cholic acid) and the presence of the other two (desoxycholic and chenodesoxycholic acids) seems not to have been appreciated. A common method for bile salt estimation was that of Irwin, Johnson and Kopola (1944), which was a modification of the method of Reinhold and Wilson (1932). This

method was specific for cholic acid and its conjugates. It did not measure desoxycholic or chenodesoxycholic acids.

Even with this imperfect method of investigation, however, certain basic features of bile salt function and metabolism became clear. For example, it was shown that protein and certain amino-acids promoted the formation of cholic acid in the dog (Ivy, 1941); Magee, Kim and Ivy (1952). The mean endogenous cholate output of chronic biliary fistula dogs was found to be approximately 100mg/Kg/24 hours by Magee, Kim, Pessova and Ivy (1952), and these workers also pointed out that in cases where cholate output fell significantly below this level 'hepatic dysfunction' could be demonstrated by other independent methods.

An entero-hepatic circulation of bile salts has been known for a long time, and Berman, Snapp, Ivy and Atkinson (1940), in an introduction to their work on biliary-duodenal fistula dogs, state that it was first demonstrated by Schiff in 1870. Berman et al (1940) confirmed the entero-hepatic circulation of bile salts in dogs and showed that, at each such circulation, about 10 - 12% of the bile salts were 'lost' or 'destroyed'.

More precise knowledge of the bile acid mechanism has been made possible by the development of a method for the total analysis of bile acid content of very small samples of bile or intestinal contents. The method, developed by Sjorvall (1956) has made it possible to determine both the glycine and taurine conjugates of the three bile acids, cholic, desoxycholic and chenodesoxycholic acid. It involves paper chromatography of the bile sample and determination of the individual bile acids as their sulphuric acid chromogens. This method, together with other procedures for separating bile acids and bile salts with column partition chromatography, for labelling bile acids with isotopes and for synthesising conjugates on a micro-scale, have been used to elucidate the problem of bile acid synthesis. The subject has been reviewed by Hazlewood (1955), Bergstrom and Borgstrom (1956) and Bergstrom (1958), and the following is a brief account of the conclusions reached by these reviewers:-

These reviewers accepted that most cells could synthesise cholesterol from acetate, and that the liver was the main site of this synthesis. The transformation of cholesterol into cholic acid was

first shown by Bloch, Berg and Rittenburg (1943) and confirmed by Bergtrom and Borgstrom (1956), who showed (in rats) that the main part of the cholesterol was degraded to cholic and chenodesoxycholic acids and that these were excreted from the liver as taurine conjugates.

The turnover of the bile acids in the rat with an intact entero-hepatic circulation was found to be about 5 mg. per day, with a total bile acid pool of 20 mg. (Lindstedt and Norman, 1956). In rats with an acute biliary fistula (i.e. no entero-hepatic circulation of the bile salts) the quantities involved have been shown to be different. Erikson (1957) showed, in a typical experiment, that on the first day of the fistula, 22 mg. of cholic and 7 mg. of chenodesoxycholic acid were excreted. But when this total was broken down to 6 hour fractions, it became evident that during the first 12 hours the main part of the bile acids present in the entero-hepatic circulation was excreted. There was then (12 - 18 hours) a minimum in the excretion rate, followed (18 - 24 hours) by a rapid rise, due to an increasing endogenous synthesis. During the second day, the total synthesis was 50 mg. cholic and

10 mg. chenodesoxycholic acid. Erikson concluded that the result of breaking the entero-hepatic circulation was a rapid increase in hepatic synthesis to more than ten times the normal daily synthesis. The mechanism which regulated the normal rate of bile acid formation in the liver has broken down in the animal with a biliary fistula. This problem was further investigated by Gustaffson, Bergstrom, Lindedt and Norman (1957), who found that by instilling bile salts at 10 mg. per hour into the duodenum of a rat with a biliary fistula, the formation of cholic acid by the liver was reduced to normal levels. They concluded that, under normal conditions, the same amount of bile salt (10 mg.) per hour reached the liver via the portal blood. This means that to keep up this rate the normal pool of 20 mg. would have to circulate between the liver and investines 10 times per day.

In man, estimates have been made of the various bile acids in gall-bladder bile. Sjorvall (quoted by Bergstrom, 1958) found the concentrations of bile acids in normal gall-bladder bile to be :-

Cholic acid	2.42 Gm/100 ml.
Chenodesoxycholic acid	2.32 Gm/100 ml.
Desoxycholic acid	1.46 Gm/100 ml.

The daily production of cholic acid in 8 normal males was found to average 0.36 Gm/day (Bergstrom, 1958) and the total bile acid production to be about 1.0 Gm/day.

The function of the bile salts is, mainly, that of an emulsifying agent in the intestinal absorption of fat. Bile salts possess the two necessary properties of a fat emulsifying 'detergent' - type molecule, namely a fat-attracting and a water-attracting part. The nuclear - CH_2 group and the methyl group are the fat-attracting portion of the molecule; the water-attracting group are the _OH, $_SO_3$, _COOH, and $_COO^-$ (Hazlewood, 1955). It has been shown by Raines and Crawford (1953) that, at physiological concentrations, the bile salt molecules will be in the form of micelles, in which the hydrocarbon (fat-attracting) part of the molecule lies at the centre of a micelles, and the polar (water-attracting) parts lie outside, in contact with the surrounding aqueous medium.

Peters (1941) suggested that bile salts influenced the active intestinal absorption of chloride. Using isolated loops of liver ileum in anaesthetised dogs, he showed that sodium taurocholate and sodium glycocholate in 1.5 Gm/100 ml. concentration,

decreased chloride absorption. Unconjugated bile salts had the same effect but at much lower concentration (0.2 Gm/100 ml.)

Strain and Marsh (1936) showed that bile salts, at certain concentrations, could depress the oxygen uptake of dog tissue slices in Ringer-phosphate-glucose medium. They observed that conjugation seemed to diminish the inhibitory effect of bile salts on tissue slice respiration.

The effect of injected bile salts on hepatic blood flow has been investigated by Grodins, Osborne, Ivy and Goldman (1941). These workers used direct current thermostromuhrs for their measurements and were concerned with the confirmation of an earlier finding that a synthetic substance, structurally resembling bile acids, markedly increased hepatic arterial blood flow. The substance was Decholin, i.e. triketo cholanic acid (ordinary bile acids are di- or tri-hydroxy cholanic acids. Grodins et al (1941) confirmed (in 42 experiments) that injected Decholin caused an increase in hepatic arterial blood flow - the increase ranged from 15 - 218%, with an average of 48% over control values. However, their results with 'natural' bile acids were not so conclusive. Sodium

cholate, for example, resulted in no change in the hepatic arterial blood flow in 11 experiments out of 27. In 10 out of 27 experiments, sodium cholate caused a decrease in hepatic arterial blood flow. In only 6 experiments out of the 27 was there an increase in hepatic arterial blood flow following the administration of sodium cholate.

4. EXOGENOUS COMPONENTS OF BILE.

Many substances which are administered to the body are found in the bile in varying quantities. Among these diverse substances, those normally occuring in bile, such as bilirubin and bile salts may be noted, as well as certain dye stuffs foreign to the body which are used in liver function tests experimentally or clinically. Other substances used therapeutically may also be found present, although they are usually not so readily identified as the dye stuffs.

Following an injection of exogenous bilirubin into the bloodstream, it disappears very rapidly from the blood, partly owing to the initial diffusion into the tissues, but when principally due to the activity of the liver cells (Dragstedt and Mills, 1937; Mills and Dragstedt, 1938). The injected bilirubin occurs

in high concentration in the bile (Green and Snell, 1928; Berman, Snapp and Ivy, 1941; Cantarow, Wirts, Snape and Miller, 1948) but as a rule the entire quantity of bilirubin administered cannot be traced in bile (Cantarow et al, 1948). Bilirubin can also be eliminated through the kidneys, but much less effectively than through the liver (Harrop, and Barron, 1931; With, 1947; Lichtman, 1949). The renal excretion, however, only begins after a certain threshold concentration in the blood has been reached. This threshold level may vary in height in different animals.

Greene and Snell (1928) found that bile salts disappear more rapidly from the blood than bilirubin after intravenous injection. Even when large doses of bile salts were used, the major part of the administered quantity was found in the bile after two hours (Josephson, Jungner and Reydin, 1938). This applies to conjugated and unconjugated cholic acid. Berman, Snapp and Ivy, (1941), found that other bile salts might be handled differently by the liver, depending upon whether or not and oxidised or not.

Since the early studies by Chronszewsky (1866) on the elimination and concentration of dye

stuffs in the bile, it has been found that a large number of these substances behave in a similar manner (see Adler, 1929; Hober, 1946). Von Mollendorff (1915) was of the opinion that dye stuffs occur in the bile more rapidly and at a higher concentration, the more readily diffusing they are.

The dependence of the transport and of the concentration of the dye upon the nature of the dye and on the cellular activity, as studied particularly in the liver by Hober and co-workers, has already been mentioned. The majority of the dyes eliminated and concentrated, often to a marked degree in the bile, are readily diffusing acidic compounds which are actively transferred through the liver cells.

Among the dye stuffs, bromsulphthalein has been used extensively in function tests of the liver. Cantarow, Wirts, Snape and Miller (1948) found similar conditions of concentration for bilirubin and bromsulphthalein in blood and bile after intravenous administration, contending that these substances are eliminated by the same mechanism. Comparable results were obtained by Hanzon (1952). Cantarow et al demonstrated, also with regard to bromsulphthalein, that occasionally only some of the administered

quantity of dye is traceable in the bile in spite of its disappearance from the blood. The colorimetric methods used for determining the quantity of dye present in the bile do not reveal the total quantity present according to Brauer, Pessotti and Krebs (1955) when compared with recovery of bromsulphthalein labelled with S^{35}. The dye may be excreted in the urine but following the standard 5 mg/Kg dose used in the clinical liver function test, this is negligible (Norcross, White and Bradley, 1951). Following liver damage, however, the loss of dye in the urine is considerably increased (Giges, Mann and Sharron, 1952). When continuous infusions of the dye are given, some of the dye is taken up by other tissues or excreted by the kidney as well as being taken up by the liver and excreted in the bile (Cohn, Levine and Streicher, 1947; Cantarow et al, 1948; and Brauer and Pessotti, 1949, 1950, ~~1955~~). However, with the reasonably low plasma concentrations of dye used clinically, this extra-hepatic loss is not significant (Bradley, Ingelfinger, Bradley and Curry, 1945).

5. THE OCCURRENCE OF BILIARY COMPONENTS IN THE BLOOD

The occurrence of biliary components in the blood partly regulates the activity in the liver cells

with which they are transported from the blood to the bile (Bayliss, 1924). This effect may be manifested in one or both of two ways; by either a change of the bile volume output or a change in the concentration of the total solids in the bile.

Bile salts may exert a different effect depending upon their nature. Sodium cholate, also conjugated with glycine or taurine produces a moderate increase in the bile volume and a simultaneous increase in the concentration of solids (Green and Snell, 1928; Berman, Snapp, Ivy, Atkinson and Hough, 1940; Brauer and Pessotti, 1952). Other bile salts may increase the bile volume output to a very different extent, sometimes very considerably (Grodins, Berman and Ivy, 1941), but the total solids may be unchanged or even lowered, as described by Berman et al (1940) and Brauer and Pessotti (1952) with regard to dehydrocholate. The bile salts exert their effect on bile formation by a direct action on the liver cells, independent of the changes occurring simultaneously in the circulation (Brauer and Pessotti, 1952).

When the sodium salt of bilirubin is administered to the blood, it has no effect on the bile

volume output (Green and Snell, 1928; Cantarow et al, 1948), but the concentration of total solids is increased through secretion in high concentration. Many other substances affect the biliary secretion with regard to the composition or volume of the bile. Such an effect has been attributed by Hober and Titajew (1928), Valdecases (1931), Hober (1932, 1946), and Hober and Moore (1939) to the fact that the substances are surface activity and disperse towards various hydrophilic colloids. These choleretic substances were found capable of increasing the bile volume and the concentration of the normal bile constituents as well as other substances, such as dyes, eliminated simultaneously. Hober et al worked with perfused frog liver. From analyses of the liver function under more physiological conditions in the intact body, it would seem that the question of whether a substance can increase the concentration, or the eliminated quantity of another substance, remains unsolved. Adler (1929) found the concentration of bilirubin in the blood of human subjects fell at the administration of bile salts and that hepatotrophic dyes were more readily traceable in the duo-

denal fluids. Cantarow and Wirts (1943) and Cantarow, Wirts, Snape and Miller (1948) found no increase in the elimination of bromsulphthalein with bile acids in dogs. The latter investigators, as well as Berman, Snapp and Ivy (1941) observed that the eliminated quantity of exogenous bilirubin was not affected by different bile acids and other choleretics. Sodium cinchopen appeared to increase the elimination of endogenous bilirubin in Cantarow et al's experiments, but this was not demonstrated in the case of bile acids.

Bromsulphthalein itself gives rise to a slight choleresis, but does not affect the elimination of endogenous bilirubin, although the excretion of exogenous bilirubin is reduced (Cantarow, Wirts, Snape and Miller, 1948). Benemid produces a marked choleresis without any alteration in the elimination of endogenous bilirubin. It, however, considerably reduces the elimination of bromsulphthalein (Goetzee, Richards and Tindall, 1958).

V. THE FLUID COMPARTMENTS OF THE LIVER

The necessary fluid compartments of the liver, namely the hepatic cell, the blood, the bile, the tissue fluid and the lymph are obviously inter-related, but in a complex manner which at present is not fully understood. However, since the mathematical model introduced in this thesis is based on the distribution of the dye bromsulphthalein within these spaces, some information regarding these compartments is considered here.

The blood and lymph:- The relationship between liver lymph and the blood plasma which traverses the liver is not precisely known, although many workers have noted the similarity in composition of these two fluid compartments. The total protein content and the albumin:globulin ratio in plasma and liver lymph have been shown to be almost identical (McCarrell, Thayer and Drinker, 1941; Brauer and Hardenbergh, 1947). Brauer (1955) also has shown that the close resemblance of liver lymph to blood plasma persists even on electrophoretic analysis of the two fluids.

In order to define more clearly the relationship between plasma and liver lymph, an attempt was made to assess the distribution space of plasma

protein and of the cellular elements of blood within the liver. The workers involved, (Gibson, Seligman, Peacock, Aub, Fine and Evans, 1946; Brauer, 1955) used albumin labelled with radioactive iodine and red blood cells labelled with radioactive Fe^{59}. They found that in the rat, the distribution space of red cells amounted to only 5% of the liver mass, whereas the iodinated albumin space amounted to about 15% of the liver mass. Brauer (1955) extended this type of investigation by making a continuous recording of the level of radioactivity in the liver following the administration of the radioactive materials. When radioactive red cells were injected, Brauer observed that radioactivity in the liver rose very rapidly to a steady plateau, which was maintained thereafter. When iodinated albumin was injected, the rise in liver radioactivity was much slower and a plateau was only gradually attained.

Brauer concluded that the spaces measured by the two labelled substances were "by no means the same" and suggested that the true intravascular space was that which contained the red cells only, and that plasma protein, not retained by the endothelial walls rapidly diffused almost throughout the entire extra-

cellular space within the liver (Brauer 1955, 1956). Manery and Bale in 1941 had previously shown that the extracellular sodium space ranged from 17 - 20% of the liver mass.

It would seem that, since the distribution space for plasma proteins is substantially the same as the extracellular sodium space, the parenchymal cells of the liver are bathed by a medium which is almost completely in equilibrium with blood plasma passing through the liver. Brauer (1955) concluded his observations as follows :- "The rapidity with which the iodinated albumin distributes itself throughout the entire space available to it, is sufficient to suggest that it is no longer permissible to interpret the hepatic circulation in terms of the flow of blood through a closed vascular tree; rather it would appear as though a new concept would have to be evolved to accomodate what appears to be an extravascular circulation of fluid, electrolytes and plasma protein in the liver".

It must, however, be pointed out finally that there is no definable anatomical counterpart of this 'extracellular space'. Even if the spaces of Disse are accepted as fact and not artefact, these

spaces could not possibly account for 17 - 20% of the liver mass.

The blood and bile :- The physiological relationship between blood and bile in the liver is also difficult to define with precision. Studies in the isolated liver involving the use of cross-over circulations and variously isotopically labelled electrolytes (ions) have strongly suggested that bile flowing from the liver is closely related, in composition, to the plasma flowing past the liver cells, rather than to the liver cells themselves (Brauer, Holloway, Krebs and Leong, 1954; Leong, Holloway and Brauer, 1955). Observations made by these workers have indicated that some substances (e.g. K^{42}) appear in bile only minutes after being added to the perfusate.

On the other hand, most of the work concerning the excretion of pigments and dyes in the liver, has shown no direct relationship between the plasma level of dye (or pigment) concentration and the level of the dye in the bile. Similarly, the time-lag between the administration of a dye and its appearance in the bile is long (Brauer, 1955).

It is possible to interpret these conflicting

observations by assuming that there are two separate relationships, as follows :-

(i) blood and bile can, under some experimental conditions, be directly related; the anatomical site where blood and bile are in close contact could be the peribiliary plexuses described by Maegraith, Andrews and Wenyon, 1949 and Maegraith, 1958. It would seem, however, that this contact only permits the exchange of electrolytes.

(ii) blood and bile are indirectly related, by means of the liver cell. The transfer of certain substances from the plasma to the bile takes place across the liver cell.

The bile and the liver lymph:- It has been noted that there exists some relationship between liver lymph and the bile. Obstruction of the common bile duct, for instance, results in the prompt appearance of bile pigment in lymph collected from the liver. Similarly bile acids appear in the liver lymph after experimental ligation of the common bile duct (Mayo and Greene, 1929). This could mean either that bile acids, after being secreted by the liver cell into the bile are then passed

into the lymph, or that the liver cell has, in fact, reversed its polarity and is secreting bile acids into the 'extracellular spaces' of the liver and bile acids then pass into the lymph. There is no direct evidence to support either view; it is only possible to conclude that liver lymph and bile may, under certain conditions, come into equilibrium with each other.

VI. DYES AND THE LIVER

The liver, like the kidney, is capable of dealing with a large variety of substances, foreign to the body, either by excreting them into the bile, changed or unchanged chemically or altering them in nature so that they are suitable for elimination in the urine. In many cases, the exact site of preference for elimination is unpredictable with our present state of knowledge. A substance may be eliminated unchanged in the urine or bile or it may in fact be eliminated in both the urine and bile, one or other of these channels however, usually being predominant. There are many foreign substances used either therapeutically or for investigation purposes, but unfortunately many cannot be adequately traced with ease, if at all. The dyestuffs and coloured compounds (the latter being loosely called 'dyes) form large and well documented groups in relation to the kidney and liver. Bromsulphthalein (Rosenthal and White, 1925), Brilliant Vital Red (Davies, Wadsworth and Smith, 1930), Sulphonic acid azo-dyestuffs (Hober, 1940), uranin (Fluorescein) (Grafflin, 1947; Hanzon, 1952, 1958), Rose Bengal (Mendeloff, 1949) for example, have all been shown to appear in the bile after injection in-

to the bloodstream.

According to Cantarow, Wirts, Snape and Miller, 1948 and Hanzon, 1952, these substances share certain physical and chemical properties with bilirubin, and they have been shown to undergo similar treatment in the liver. Bilirubin is the normal endogenous substance to be taken up by the liver cells and secreted into the bile. It would, therefore, seem probably that these foreign substances would also follow the usual pathway of excretion. A study of any one of these substances should, therefore, apply in general to them all and indicate the normal excretory pathways of the liver. Uranin (Fluorescein) is, with the aid of ultra-violet light, visible in very low concentrations and was on this account chosen by Hanzon for his studies in the rat liver. Using uranin, Hanzon (1952, 1958) was able to trace the excretory pathway of this dye through the liver from the plasma to the bile.

A brief outline of Hanzon's work is as follows :-

The abdominal cavity of the anaesthetised rat was opened and the liver fixed on a microscopic

stage. The exposed liver surface was irrigated with fluid which was physiological as regards temperature, salt content and colloid osmotic pressure. Ultra-violet light was transmitted through the objective lens by means of a vertical illuminator. A cannula was placed in the bile duct for the collection of bile samples; and another cannula placed in the femoral vein for the injection of uranin and the collection of blood samples. Hanzon maintained that the rats under these conditions remained in a stable and standard condition for up to four hours, and that at no time was any structural damage ever observed of the liver during the period of the experiments.

After the rapid injection intravenously of uranin, its typical fluorescence was seen in the blood sinusoids of the liver within a few seconds. About twenty seconds later, it was detectable in the liver cells, their normal blue fluorescence gradually passed into a stronger, greenish-yellow colour. Fifty seconds after the first appearance of dye in the sinusoids, the bile capillaries began to show the typical fluorescence. This fluorescence became more intense during the next five to ten minutes and

then gradually decreased. After thirty to sixty minutes there was no longer any trace of the substance in the liver cells and after two to three hours it was no longer visible in the bile canaliculi. The concentration curve of uranin in the collected bile samples ran parallel with the observed fluorescence intensity in the bile capillaries.

In the liver cells themselves, Hanzon observed the fluorescence to be distributed over the whole cytoplasm, without being concentrated in any particular structure, such as the Golgi apparatus. This agrees with the findings of Davies et al (1930) concerning Brilliant Vital Red. These workers observed that cells which secreted this substance did not themselves show high concentrations or even granule formation.

Hanzon concluded from his own observations that the liver surfaces were primarily involved in the transfer of fluorescent material from the plasma to the bile. The liver cell surface 'facing' the sinusoid took up the substance and rapidly passed it to the cell surface 'facing' the bile capillary for excretion (Hanzon, 1958). He also suggested that the reason for the relatively slower excretion of

dye from the liver cell to the bile (and hence temporary storage effect) was that of these two 'facing' surfaces, the one facing the bile capillary was relatively much smaller than that facing the sinusoid.

This work of Hanzon in the rat liver is in substantial agreement with that of Grafflin (1947) on frog liver. Grafflin observed the passage of uranin from plasma, through the liver cell, and into the bile, and concluded that his observations offered no objective evidence "for the existence of any route, other than through the cytoplasm of the liver cell, for the transfer of material from blood in the sinusoids to bile in the canaliculi".

Rose Bengal, another fluorescent material, has been shown by Mendeloff (1949) to be transferred from plasma to bile only by way of the hepatic polygonal cells. After the intravenous injection of a quantity of Rose Bengal, Mendeloff found that the liver biopsy specimens, taken one to fifteen minutes later, showed the typical fluorescence in the liver cells. His observations were mainly in the rabbit liver, but he noted that in two human liver biopsy specimens, the fluorescence of the injected Rose

Bengal was as in rabbit liver, limited to the hepatic polygonal cells.

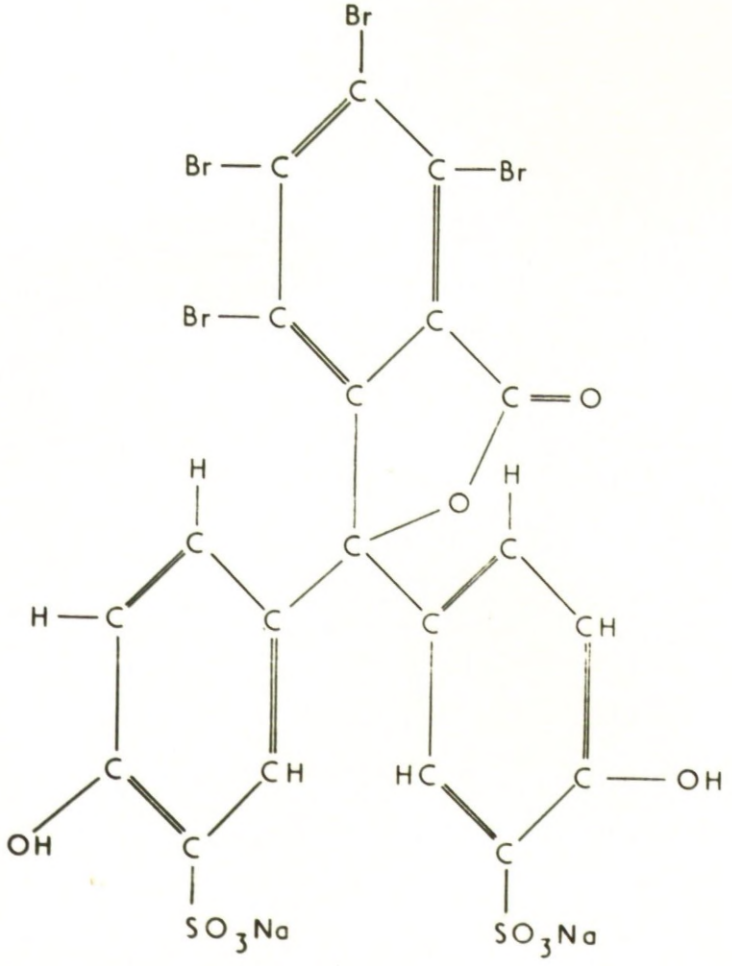
Bromsulphthalein is not fluorescent, and there is consequently no direct visual proof of its presence in the liver cell in the process of transfer from plasma to bile, but, as mentioned above, it would seem reasonable to expect it to follow a similar pathway to the fluorescent materials.

VII. BROMSULPHTHALEIN AND ITS DISTRIBUTION IN THE BODY

Figure 17.

The formula of bromsulphthalein.

"BROMSULPHTHALEIN"



1. BROMSULPHTHALEIN.

The dye commonly known as bromsulphthalein, bromsulphalein or BSP is the di-sodium salt of phenol tetrabromsulphthalein sulphonate, and was introduced into the study of liver function in 1925. Structurally, it is di-sodium 3:4:5:6 tetra-brom-di(hydroxy-3-sulpho-phenyl) phthalide and it is depicted in Figure 17. Its molecular weight is 838.1. It is prepared by the interaction of phenol and tetrabromophthalic acid or the anhydride of the latter, followed by the sulphonation of the product and conversion to the di-sodium salt. It is a white crystalline powder, which is odourless and bitter to taste. It is hydroscopic, soluble in alkali but insoluble in alcohol or acetone.

In 1925, Rosenthal and White reported their observations on a large number of phthalein compounds which had been suggested as useful in the estimation of liver function because they were all excreted in high concentration in the bile of animals. Of the phthaleins so studied, bromsulphthalein, which had been synthesised by White, was found to be the most useful and sensitive in the estimation of liver damage.

2. SOME OF THE PROPERTIES OF BROMSULPHTHALEIN.

Rosenthal and White (1925) also found that bromsulphthalein was excreted in rabbit bile to the amount of 60 - 90% of the injected quantity in the first hour, after an intravenous injection of 5mg/Kg/body weight.

In the dog, bromsulphthalein is transferred from the blood to the bile equally rapidly. Mills and Dragstedt (1938) found that after an intravenous injection of bromsulphthalein (2mg/Kg/body weight), 85 - 90% of the dye had disappeared from the blood in five minutes. Wirts and Cantarow (1942) showed that bromsulphthalein was detectable in bile within minutes of the injection into the bloodstream, and Cantarow, Wirts, Snape and Miller (1948) showed that between 50 and 80% of the injected dye was detectable in bile within one hour of the injection having been given.

In 1950, Brauer and Pessotti attempted a quantitative estimate of the liver's ability to remove bromsulphthalein from the plasma. Instead of giving bromsulphthalein as a single injection, these workers used a continuous infusion intravenously of the dye. Using dogs they measured the concentrations

of bromsulphthalein in the plasma and bile. They found that for normal dogs receiving an infusion of bromsulphthalein at a rate of less than 0.16 mg/Kg/min. the amount extracted per minute from the blood was 15 - 0.69% of the quantity present in the blood at that time, and in normal dogs with infusion rates higher than this level, they observed decreasing bromsulphthalein extraction rates. In these experiments, with high infusion rates they found that the concentration of bromsulphthalein did not exceed 9.3 - 0.9 Gm/litre. Brauer and Pessotti also drew attention to the fact that only 55.8% of the bromsulphthalein which was removed from the blood could be accounted for in the bile and liver. They thought, however, that the low rate of recovery could be partially explained by deficiencies in their methods of estimation of the dye colourimetrically.

Brauer, Pessotti and Krebs (1955) investigated this discrepancy later using radio-active labelled S^{35}. It was found that the use of S^{35} bromsulphthalein considerably narrowed the gap between the amount of bromsulphthalein infused i.e. the amount extracted from the blood at equilibrium and the amount recovered

from the liver and bile. The bile invariably contained more S^{35} than could be accounted for by colourimetric estimation and it was therefore concluded that the dye probably went under some sort of metabolic transformation before being excreted into the bile.

The same group of workers confirmed this conclusion a few years later (Krebs 1958; Krebs and Brauer 1958) when they investigated the chemical nature of bromsulphthalein excreted into the bile by the rat, pheasant, chicken, cat, dog and cow. They used a chromatographic method, sensitive for the separation of phthalein dyes and for the separation of bromsulphthalein derivatives in bile. The optical densities of the derivatives were then measured in a spectrometer. They found that in rat bile, for example, there are three distinct bromsulphthalein compounds which resembled bromsulphthalein in colour but were separable by chromatography. They all had almost identical spectro-photometric absorption curves, but their extinction coefficients differed from that of pure crystalline bromsulphthalein. They called these derivatives bromsulphthalein-I,-II and -III.

Their investigations showed that bromsulphthalein-I was, in fact, the same as pure crystalline bromsulphthalein, and that bromsulphthalein-III was the compound found in the bile in the greatest quantity.

The three compounds which Krebs and Brauer isolated from rat bile, were isolated and purified and then reinjected into other rats. When bromsulphthalein-I was injected, all three compounds appeared in the bile; when bromsulphthalein-II was injected, only bromsulphthalein-II was found in the bile and when bromsulphthalein-III was injected a mixture of bromsulphthalein-II and -III were found in the bile. The bromsulphthalein derivatives -I, -II and -III were present not only in the bile but were also demonstrated in the blood plasma following an injection of pure bromsulphthalein. Krebs (1958) reported that eight minutes after an injection of bromsulphthalein, the proportion of bile-type derivatives in plasma was small (about 2%), but that it rose steadily, so that thirty minutes after the injection the proportion was about 75% of the total bromsulphthalein present. At this stage, however, the total quantity concerned was very small despite the high percentage present, since only about 1% of

the dose injected remained in the plasma.

These workers have shown also that the rate of removal of bromsulphthalein-II and -III from the plasma was less than the rate of removal of pure bromsulphthalein in the rat. Since both bromsulphthalein-II and -III are present in the plasma after the injection of pure bromsulphthalein, it would be expected that the rate of total removal of dye from the plasma would show a gradual decrease, and Krebs was able to show that this was the case by observing the plasma clearance curves. The chemical nature of the bromsulphthalein derivatives has not yet been made clear. Krebs and Brauer (1958) suggest that it is probably not a glucuronide. The presence of bromsulphthalein derivatives in human bile after an injection of pure bromsulphthalein was shown by Grodsky, Fanska and Carbone (1958). Their technique was not unlike that of Krebs - separation of the derivatives by paper chromatography using independent solvent systems (dioxane-water and tert-butanol-water). Two fractions were first described in human bile, one identical with bromsulphthalein and a second which was an altered bromsulphthalein compound and which represented 65 - 75% of the total quantity in the bile.

The second fraction was analysed further and shown to contain three distinguishable bromsulphthalein compounds (Grodsky, Carbone and Franska, 1959a; 1959b), which were called bromsulphthalein-A, -B, -C. Of these three, bromsulphthalein-A was usually the most abundant in bile as was bromsulphthalein-III (in rats) of Krebs. Only trace amounts of bromsulphthalein-A and -B were found in the plasma of normal humans. The three compounds had absorption spectra identical with that of pure bromsulphthalein, but with different extinction coefficients. Grodsky and his colleagues did not think that the bromsulphthalein derivatives they found were glucuronides or ethereal sulphates. They suggested that their bromsulphthalein-A might be bromsulphthalein conjugated with cysteine or glutathione.

The work of Krebs and Grodsky gives reasonable grounds for believing that bromsulphthalein is altered in its passage through the liver and is excreted into the bile as a complex or conjugate, the precise nature of which remains unknown at present. That this alteration in bromsulphthalein is brought about during the process of excretion from the liver cell into the

bile and not during the process of simple absorption into liver protein at the blood:liver cell surface is clear from the observations of Brauer and Pessotti (1949) Investigating certain aspects of the uptake bromsulphthalein by incubated rat liver slices, they noted that the uptake of bromsulphthalein proceeded normally even in the presence of CN^- , Fe^{++} , and Hg^{++} and concluded that no metabolic process was involved in such an uptake. Further, the dye, taken up by the slices in vitro could be recovered to the extent of 90 - 95% in a form which was indistinguishable from pure bromsulphthalein i.e. uptake by the liver did not cause any observable change in the bromsulphthalein molecule.

It was also observed by Brauer and Pessotti that other tissues, such as muscle and kidney were capable of taking up bromsulphthalein from solution nearly as effectively as the liver slices. When, however, the living liver was perfused with a solution containing bromsulphthalein, it showed itself to be much more efficient in the removal of bromsulphthalein than any other tissue. Brauer and Pessotti stated that in their view the unique position

of the liver in respect to the elimination of bromsulphthalein was based, not on any special affinity of the liver cells for the dye, but rather on the ability of the liver to 'desaturate' itself by excreting into the bile bromsulphthalein which it had removed from the perfusing fluid.

3. THE PROTEIN BINDING PROPERTIES OF BROMSULPHTHALEIN

It has been demonstrated in vitro that bromsulphthalein is absorbed into incubated liver slices, and its subsequent removal has been postulated in the excretion of bromsulphthalein in vivo. It is therefore necessary to review the protein binding properties of bromsulphthalein at this stage. Since, it is can be shown that injected bromsulphthalein is bound to plasma protein , it would be reasonable to suppose that binding to liver protein is at least part of the process of 'uptake' of bromsulphthalein by liver slices and the perfused living organs. It has been shown that many dyes when injected become bound to plasma proteins and do not remain entirely free, that is in the unbound state. The dyes T1824 (Evans Blue), D1836 (Trypan Blue), D1824 (Niagara

Sky Blue 6B) have been exclusively studied in this regard by Rawson (1942) and Gregersen and Rawson (1952). These dyes, as well as others, have been shown to combine with plasma albumin and notable differences in the tendency of these dye albumin complexes to dissociate have been demonstrated. Some were found to be weakly bound to protein and dissociated easily. Others were strongly combined as protein and the dissociation was slow. For example, Trypan Blue (T1836) was shown to be only weakly attached to protein and when it was injected into the bloodstream its disappearance from the plasma was rapid. On the other hand, Evans Blue (T1824) combined strongly with plasma albumin and its disappearance from plasma and its appearance in bile was extremely slow. Concentration of T1824 in bile was always less than the concentration in the plasma. Miller (1947) investigated the excretion of this substance and found that the maximum concentration of dye in the bile was not reached until 60 - 90 minutes after the injection. Only 2% of the quantity of dye injected was found in the bile. The excretion of Trypan Blue (T1836) into bile was investigated by Hober and Titajew (1930). Trypan Blue (weakly bound

to protein) passed rapidly into the bile with concentrations between 5 and 20 times the concentration of dye in the plasma. There is thus evidence to show that some dyes are bound to protein and that the strength of binding between dye and protein is an important factor in the removal of the dye from the plasma by the liver and its subsequent excretion into bile. The work of Hanzon (1952) has already been referred to regarding the visual proof that dye binding is an important factor in the excretion of uranin (Fluorescein) by the perfused rat liver. When the rat liver was perfused with a saline solution containing uranin (1 mg/100 mls.) Hanzon observed that the hepatic cells were the site of an intense fluorescence. The 'free' dye in the perfusate had penetrated the liver cell and had become bound to liver cell protein and produced a more marked fluorescence within the liver cell than that observed in the liver sinusoids. Hanzon then perfused rat liver with blood containing uranin 2 (2 - 4 mgms./100 mls.) and observed only a feeble fluorescence within the liver cells. The presence of protein in the perfusate had reduced the amount of 'free' dye available to the perfusate. Less dye under these circumstances

penetrated the liver cells, and less was bound to liver cell protein. Since Hanzon has also shown that uranin (Fluorescein) is transferred by the liver from plasma to bile in the intact rat, it is clear that the first stage of this transfer must involve a transfer of dye from combination with plasma protein to combination with liver cell protein.

The combination of bromsulphthalein with plasma albumin has been demonstrated by Brauer and Pessotti (1949). Using isolated rat livers they observed the liver removed less bromsulphthalein from a perfusate containing bromsulphthalein and albumin (5 mgms./100 mls.) than from a perfusate of bromsulphthalein and saline alone. Similarly with incubated liver slices, more bromsulphthalein was taken up by the liver slice when the incubation medium was protein-free than when it contained plasma albumin. Brauer and Pessotti therefore concluded that the uptake of bromsulphthalein by the liver was due to a change of combination of dye from a dye plasma albumin complex to a dye liver cell protein complex.

Robinson (1943) and Ingelfinger, Bradley,

Mendeloff and Kramer (1948) have demonstrated the in vitro binding of bromsulphthalein to protein. These workers showed that bromsulphthalein dissolved in saline dialysed rapidly through a cellophane membrane, but dialysis was prevented by the addition of serum to the bromsulphthalein solution. Then by the use of salting-out techniques, they demonstrated that most of the bromsulphthalein was found bound to the albumin fraction.

4. THE TRANSFER OF DYE FROM PLASMA TO BILE.

Experimentally, it can be demonstrated that soon after an injection of bromsulphthalein the dye is present in the plasma within the liver cell and in the bile. The bromsulphthalein in the liver cell is, so to speak, in transit or in temporary storage awaiting excretion into the bile. The use of the term 'storage' seems to have been first used by Cantarow and his colleagues (in 1948) who observed a time-lag between the disappearance of bromsulphthalein from plasma and its appearance in bile, and they suggested that during this time-delay, bromsulphthalein was stored within the liver cell. The word 'storage' however, seems to suggest a certain dis-

engagement from the actual process of transfer from plasma to bile and in this sense is not an especially useful expression. The maintenance of high secretion rates of bromsulphthalein by the liver demands a constant load or store of bromsulphthalein within the cell. This has been shown by Brauer and his colleagues (1955) in isolated perfused rat livers. The liver was first perfused with a bromsulphthalein solution until a constant amount of bromsulphthalein was recovered in the bile. The liver was then changed to a perfusion medium which contained no bromsulphthalein. At once, the amount of bromsulphthalein excreted in bile began to fall, presumably because the store or load in the liver cells was itself falling, i.e. the amount of bromsulphthalein in the liver cell acts in this sense as a head of pressure resulting in the flow of bromsulphthalein at a certain rate. It has been mentioned previously that the ability of the liver cell to secrete certain substances is a limited one, that is to say, there is a limit to the amount of substance which can be transferred from plasma to bile in unit time. Hanzon (1952) showed that this was the case with bilirubin, for he observed that if

bilirubin was given in increasing doses intravenously to the rabbit, the concentration in bile did not exceed 1500 mgms./100 mls., and there was no notable change in bile volume. With regard to bromsulphthalein, Brauer and Pessotti (1950) have shown in the dog that the concentration of bromsulphthalein in the bile attains a value limited to 9.3 ± 0.9 gms./litre. Mason, Hawley and Smith (1948) have shown that the maximum amount of bromsulphthalein which can be excreted by the dog liver is 0.44 mgms./Kg./min.

This upper limit of the ability of the liver to excrete a substance into bile has been called 'the maximum excretory capacity of the liver' and it has been shown to be remarkably constant under a variety of conditions in those animals in which it has been measured repeatedly (Lewis, 1950). The process of bromsulphthalein transfer from plasma to bile by way of the liver cell is therefore not a simple process. The work, observations and opinions that have already been referred to seem to suggest that there are at least five stages in the process of bromsulphthalein transfer - (a) bromsulphthalein binding to plasma albumin (b) bromsulphthalein binding to the liver cell protein (c) storage in the liver cell

(d) chemical conversion or metabolism to give bromsulphthalein derivatives and (e) excretion into the bile of the various bromsulphthalein derivatives, and besides these processes there are other factors which may be concerned in the distribution of bromsulphthalein between the blood, liver and bile. Loss of dye to other tissues than the liver has to be considered, the known entero-hepatic circulation of bile salts has to be assessed in relationship to bromsulphthalein, the general physiological conditions which may effect the animal or individual as a whole as well as the liver, have all to be considered. The basic stages, however, of the transfer of bromsulphthalein from plasma to bile is not as likely to be effected as is the quantity or rate of dye excretion into the bile.

5. THE ENTERO-HEPATIC CIRCULATION OF BROMSULPHTHALEIN

Any bromsulphthalein excreted in the bile would in the intact animal eventually reach the duodenum and if it were significantly reabsorbed into the portal blood stream this could alter the bromsulphthalein concentration of blood collected at the periphery and

so change the picture of dye disappearance from the plasma. Lorber, Oppenheimer, Shay, Lynch and Siplet (1953) have shown in dogs that bromsulphthalein can be absorbed from the duodenum and result in low but significant levels of dye in hepatic vein, portal vein and peripheral arterial blood. They also recovered dye from the bile. However, the amount given intra-duodenally in these dogs was large (1 gram of bromsulphthalein, i.e. equivalent to 50mgms./Kg./body weight) and under ordinary conditions of bromsulphthalein usage no such large amount would be available at any time in the duodenum. A more reasonable dose of bromsulphthalein was used by Stone (1953) in anaesthetised cats and dogs. The dose of bromsulphthalein was 3 - 10 mgms. of bromsulphthalein /Kg. body weight, which was instilled into the duodenum. Bromsulphthalein concentrations were measured in blood from portal vein and carotid artery as well as in the bile. Stone found no bromsulphthalein in the portal vein or carotid artery at any time. He did, however, find bromsulphthalein in significant concentration in the bile. The bromsulphthalein first appeared in bile about 20 - 40 minutes after the instillation of the dye. It seems

then, that there is an entero-hepatic circulation of bromsulphthalein under these conditions, but since the small amount that must have been present in the portal vein was not measurable by Stone's methods, it does not seem likely that such a re-circulation of dye can significantly alter the general picture of dye disappearance from plasma. Stone used animals in which the gall bladder had been removed and the common bile duct cannulated. In the intact animal receiving an intravenous injection of bromsulphthalein, the gall bladder would presumably retain most of the bile produced during, say, an experimental period of one hour, and the amount of bromsulphthalein reaching the duodenum would be nil or almost nil. This point, however, does not seem to have been investigated.

In man, Lorber and Shay (1952) found that after the intra-duodenal instillation of bromsulphthalein (2 mgms./Kg. and 5 mgms./Kg./body weight) there was a detectable level of bromsulphthalein in the plasma. On the other hand, Owen (1951) was unable to detect any bromsulphthalein in the blood of 25 human subjects after intra-gastric or intra-duodenal administration. The difference in the

results of these two investigations probably rests on the methods of bromsulphthalein estimation. The blood concentrations of bromsulphthalein were evidently low and were measurable by one method but not the other. However, the entero-hepatic circulation of bromsulphthalein in man is probably of little importance when a single injection of bromsulphthalein is given and the rate of dye disappearance from the plasma is measured. In these circumstances, most of the injected dye would have left the plasma in 45 minutes and there seems no reason to suppose that during this time any significant amount of bile would have entered the duodenum.

6. LOSS OF BROMSULPHTHALEIN TO TISSUES OTHER THAN THE LIVER

Although there is sufficient evidence for believing the liver is primarily concerned in the removal of bromsulphthalein from the plasma it remains to be shown that other organs and tissues do not remove bromsulphthalein from the plasma, or that if they do then the amount of dye removed by them is small.

If bromsulphthalein were removed from plasma by tissues other than the liver, one would expect to

find a measurable difference in concentration of bromsulphthalein between the arterial blood and the tissue concerned in such removal, Selkurt (1953) put this to the test. He administered bromsulphthalein to anaesthetised dogs by continuous intravenous infusion (0.070 mgms./Kg./min) and took frequent blood samples from the following sites - a peripheral artery, jugular vein, hepatic vein and pulmonary artery, and a peripheral vein of the fore-limbs and hind-limbs. No differences in bromsulphthalein concentration were found between the arterial blood samples and the samples from the limb veins. The jugular vein concentration was actually a little higher than the peripheral arterial concentration. The hepatic vein blood concentration was, of course, lower than the arterial blood concentration by about 42%. Selkurt concluded that in these experiments there was no significant extra-hepatic removal of bromsulphthalein from plasma. The higher concentration of bromsulphthalein in the jugular vein probably represented a mixture with lymph bourne bromsulphthalein from the thoracic duct. It is important to record that the plasma levels of bromsulphthalein in Selkurt's experiments was never higher than 2 mgms./100 mls. of

plasma. The amount of 'unbound' dye was therefore small and the amount available for simple diffusion into interstitial fluid also small. Selkurt's findings are in agreement with those of Pratt, Burdick and Holmes (1952) who also used continuous intravenous infusions of bromsulphthalein in dogs. Their approach however, was an indirect one. They measured the hepatic blood flow using bromsulphthalein and an application of the Fick principle and found that, although different rates of infusion were used, the measurement of hepatic blood flow was independent of the rate of infusion. If bromsulphthalein did leave plasma by routes other than the liver, then the higher the plasma concentration of bromsulphthalein, the more would be lost by these routes and the value obtained for hepatic blood flow would vary as the plasma level varied. Since they found that the measurement of hepatic blood flow did not vary with either the rate of the infusion or the plasma level of bromsulphthalein, they concluded that losses to tissues other than the liver must have been insignificant. In these experiments the rate of infusion of bromsulphthalein did not exceed 0.2 mgms./Kg./min., and the plasma concentrations of bromsulphthalein did not

exceed 2 mgms./100 mls. In a few dogs of their series, Pratt et al (1952) measured the rate of flow in bromsulphthalein concentration of thoracic duct lymph. The highest concentration did not exceed 0.5 mgms./100 mls. and the mean flow rate was 0.77 mls./min. In one hour then, using the meximum lymph concentration observed, enough bromsulphthalein would be returned to raise the plasma concentration by only 0.04 mgms./100 mls. When plasma levels are maintained at or below 2 mgms./100 mls. for one to two hours by means of a continuous intravenous infusion, the loss of bromsulphthalein to sites other than the liver and again of the bromsulphthalein from the thoracic duct lymph seems to be small and probably unimportant.

An attempt has been made to measure extra hepatic losses of bromsulphthalein by observing the rate of disappearance of bromsulphthalein from the bloodstream of dogs whose liver has been removed. This would be a direct and straightforward method of investigation, were it not for the gross disturbance of circulation which follows hepatectomy, for, as well as many other changes, the cardiac output is reduced, oxygen consumption is decreased and the carbon dioxide content of the blood is decreased.

Conclusions based on measurements of the rate of flow of bromsulphthalein from the plasma under these unphysiological conditions cannot be usefully correlated to the normal physiological state that exists in the normal intact animal, since Cohen, Levin and Stricher[5] (1947) removed not only the liver but the kidneys and the whole gastro-intestinal tract of a series of dogs. These workers then measured the rate of removal of bromsulphthalein from plasma after single injections and continuous infusions of bromsulphthalein. They found that in their eviscerate preparations injected bromsulphthalein was removed from plasma but at a much lower rate than in the intact animals. In the intact animal, the concentration of dye in the blood twenty minutes after its injection was usually less than 10 mgms.% of that at one minute after injection. In the eviscerated dogs the concentration at one hour after injection was 45% of that at one minute after injection. This means that in the eviscerated, liverless dog, plasma concentrations or bromsulphthalein were greater than 5 mgms./100 mls. for a long time (45 - 60 minutes). In the intact dog, on the other hand, concentrations about 5 mgms./100 mls. were present for little more than five minutes. Persistent

high concentrations of bromsulphthalein in plasma would clearly favour the transfer of dye to, say, the interstitial fluid of skeletal muscle (the only large site remaining for the removal of bromsulphthalein in the wholly eviscerated dog) and the higher the plasma concentration, the greater would be the quantity of bromsulphthalein so transferred. In the intact animal, since plasma concentrations of bromsulphthalein are usually well below 1 mgm./100 mls. within 10 - 15 minutes of injection of the average dose (5 mgms./Kg./body weight) it can be only during this initial period that any significant extra hepatic loss can take place.

Loss of bromsulphthalein via the kidney is, under normal circumstances, small. Norcross, White and Bradley (1951) studying the bromsulphthalein liver function test with particular regard to renal excretion found that in human subjects the urinary excretion of bromsulphthalein ranged between 0.2 and 1.9% of the dose given 5 mgms./Kg., the average being 1.1%. With larger doses and continuous infusions of bromsulphthalein, this loss would obviously be greater but even in hepatectomised animals receiving a continuous infusion of bromsulphthalein, the loss in

urine has only been found to average 0.06% of the amount injected (Horvath, Hutt, Knapp and Werner (1953); Richards, Tindall and Young (1959) found less than 1% of the injected amount (5 mgms./Kg./body weight as a single intravenous injection) was excreted via the kidney in anaesthetised dogs.

7. THE EFFECT OF GENERAL PHYSIOLOGICAL CONDITIONS ON THE ABILITY OF THE LIVER TO REMOVE BROMSULPHTHALEIN FROM THE BLOOD-STREAM

A great deal of work on the distribution of injected bromsulphthalein within the body has been done on anaesthetised dogs which have suffered a greater or lesser degree of surgical trauma. It is necessary, therefore, to know to what extent departures from the normal physiological state of an animal will affect the ability of the liver to deal with injected bromsulphthalein. Brokaw and Penrod (1949) showed that a fall of temperature has an effect on the rate of disappearance of bromsulphthalein from the plasma. They gave bromsulphthalein (5 mgms./Kg) as a single injection to anaesthetised dogs who were then cooled by immersion in a water bath. Blood samples were collected by a catheter from the right side of the

heart at 6, 12, 20 and 30 minutes after the injection. These workers observed a reduction in the rate of bromsulphthalein disappearance from the plasma when the immersed dogs' temperature was 35°C. and an even more marked reduction when the temperature was 30°C.

The effect of carbon dioxide retention on the removal of bromsulphthalein from the plasma was studied by Holmes and Barnhart (1958). They recorded that exposure of anaesthetised dogs to raised carbon dioxide tensions resulted in prolonged retention of the dye in the plasma, i.e. removal from the blood plasma was delayed. Anoxia was ruled out as a cause since normal oxygen tensions were maintained. The effect of surgical procedures on the rate of bromsulphthalein disappearance from plasma was reported by Drill, Annegers, Snapp and Ivy (1945). After chronic biliary fistulae had been made in a group of 14 dogs, the rate of bromsulphthalein removal from the plasma was observed to be less than normal in all the dogs. Drill et al suggested that this abnormal retention of dye was due, not only to the operation itself, but also to a change in the excretion of bromsulphthalein associated with the dilatation of the bile duct system which was known to

follow cholecystectomy (cholecystectomy is part of the Rous - McMaster technique of preparing an animal with a chronic biliary fistula).

VIII. THE CLINICAL AND EXPERIMENTAL USES OF BROMSULPHTHALEIN

Unless stated otherwise, the main sources of information for this section have been the text books by Cantarow and Trumper, 1955; Popper and Schaffner, 1957; and Sherlock, 1958.

The experimental demonstration that the liver was the organ primarily responsible for the removal of injected bromsulphthalein from the bloodstream was followed by attempts to use the dye in the assessment of liver functions. If the normal liver of the experimental animal could remove bromsulphthalein from plasma then it was reasonable to suppose that the abnormal diseased liver might lack this ability in part or in entirety. Several methods have been suggested for the study of disturbed liver function using the dye, bromsulphthalein. These, however, will be dealt with in a subsequent section and the general principles of the test will be dealt with here.

1. THE PHYSIOLOGICAL BASIS OF THE BROMSULPHTHALEIN TEST

Bromsulphthalein is taken up by the hepatic polygonal cells and excreted in the bile. None can be demonstrated in ascitic fluid and little is removed by the extra hepatic tissues in the presence of a normal

liver. The rate of disappearance of bromsulphthalein from the blood indicates the activity of the liver and determinations of biliary bromsulphthalein are chiefly used for physiological studies. Under normal conditions, the disappearance rate from the bloodstream is constant. Bromsulphthalein in some instances of liver disease is associated with a progressive decrease in clearance or disappearance rate, because the capacity of the hepatic removal mechanism is reduced by liver damage. In other instances, the clearance is low but constant, suggesting an impaired blood flow through the hepatic lobule rather than liver cell damage. As a result, the uptake of the dye by liver cells depends not only on the functional capacity but also on the efficiency of the circulation. Exercise, standing and fever increase bromsulphthalein retention. The influence of the circulation upon hepatic bromsulphthalein uptake allows its use in the estimation of hepatic blood flow, or splanchnic blood flow, by measuring the hepatic extraction by catheterisation of the hepatic vein.

The applicability of bromsulphthalein to the study of liver function is based on the assumption

that its removal from the circulation is accomplished virtually exclusively by the liver. Studies in hepatectomised animals have indicated that other organs besides the liver can remove bromsulphthalein from the bloodstream, but, in the presence of a normal liver, probably not more than 5% is removed by extra hepatic tissues at the outside. If there is marked retention of the dye in the plasma, as much as 10% may be excreted in the urine and considerably more in the presence of albuminuria (the dye is bound to the plasma albumin). It is also claimed that another source of error in interpreting results occurs because some of the bromsulphthalein excreted in the bile is reabsorbed into the portal circulation, a small amount passing back into the systemic circulation, (Lorber and Shay, 1950, 1952). The original concept of the significant participation of the reticulo-endothelial system in the primary removal of bromsulphthalein from the bloodstream has not received support. (Zilversmit and Shore, 1954). Later work by Brauer, Pessotti and Krebs (1955) has confirmed that the reticulo-endothelial system plays no part at all in the elimination of bromsulphthalein. There seems little doubt, that, under clinical conditions, the

liver is the organ principally involved in this process, the relatively minor factors of extrahepatic removal, enterohepatic circulation and renal excretion do not significantly affect the interpretation of results of the bromsulphthalein test in terms of liver functional efficiency.

2. THE PRACTICAL METHODS USED FOR THE BROMSULPHTHALEIN TEST

Agreement is lacking regarding the optimal dose and time of sampling. The following have been suggested :- 2.0 mg. per Kg. body weight dose with readings at 20 minutes (Mateer, Baltz, Comanduras, Steele and Brower, 1947) or 30 minutes (Lorber and Shay, 1952), 5.0 mg. per Kg. with readings at 15 (Moses, Critchfield and Thomas, 1948), 30 minutes (Helm and Machella, 1942) and 45 minutes (Mateer, Baltz, Marion and MacMillan, 1943), as well as an intermediate dose of 4 mg. per Kg. The smaller dose originally suggested by Rosenthal and White (1925) has the advantage of a zero reading in normal cases and it reduces any possible effect of an entero-hepatic circulation. The 5.0 mg. per Kg. dose is most widely used because of the increased burden on

the liver and improved chances of testing its ability (Maconald, 1939). A 4.0 mg. per Kg. dose with readings after 45 minutes is probably optimal, in that the disappearance curve is linear under normal circumstances. Greater sensitivity is obtained by making serial determinations of the plasma concentration of bromsulphthalein with the plotting of a curve of dye removal from the bloodstream. These curves may reveal evidence of mild hepatic dysfunction causing a temporary delay in clearance, (Goodman, 1952; Goodman and Kingsley, 1953; Macdonald, 1939; Mateer, Baltz, Marion and MacMillan, 1943). Although this is theoretically more sound, it is seldom resorted to in clinical practice where only one sample is usually taken.

In the original bromsulphthalein (2.0 mg./Kg. dose) test (Rosenthal and White, 1925, the disappearance of dye was determined by comparing the concentration 30 minutes after injection with that immediately after complete mixing of the dye in the bloodstream. The results were recorded as a percentage retention, the first specimen being considered 100%. Subsequently, a solution containing 4.0 mg./100 ml. was taken as the 100% standard, since (based on the fact that the plasma volume averages 50 ml./Kg./body weight, the initial

concentration, if instant and complete mixing occurred, would be 4.0 mg./100 ml. using a 2.0 mg./Kg./body weight dose of bromsulphthalein) this eliminated the necessity of a sample immediately after mixing. Since changes of plasma volumes are ignored with this standard, readings more logically should be recorded in terms of concentration rather than as percentage retention. Using the 5.0 mg./Kg. dose and the same assumptions, 10.0 mg./100 ml. is taken as the 100% standard. More recently spectophotometric readings have been used to eliminate much of the interference due to blood and bile pigments which had previously caused errors in estimation of the dye (Gaebler, 1945). The bromsulphthalein test as a result can be combined with other liver function tests such as the galactose and hippuric acid tolerance tests (Zieve, Hill and Nesbitt, 1950; Cohen, Althausen, Uyeyama and Treager, 1951).

3. THE TOXICITY OF BROMSULPHTHALEIN.

In the doses employed, bromsulphthalein is practically non toxic, even in the presence of severe liver damage. The solution used is irritating to the tissues and may cause pain and inflammation if a significant amount is injected outside the vein. It may

also produce thrombophlebitis at the site of the injection. Occasional toxic reactions have been observed, including fever, nausea, vomiting, vertigo and syncope. Allergic reactions have been reported, especially if serial tests are performed and also in a few instances, severe anaphylactoid reactions, with severe cardio-respiratory manifestations and shock. Although these are extremely unusual, care should be exercised in performing this test in subjects with an allergic background (Chambers and Moister, 1948; Morey, Gabuzda and Scudamore, 1949; Roth, 1950).

4. SIGNIFICANCE OF ABNORMAL BROMSULPHTHALEIN RETENTION

In normal subject, using any of the doses given above, the retention varies between 0 and 6 per cent. Abnormal values in hospital control patients occur less frequently than with any of the other liver function tests, and the tests can be used in children (Mollinson and Cutbush, 1949). Inasmuch as the hepatic excretory mechanisms for bilirubin and bromsulphthalein are apparently similar (Cantarow, Wirts, Snape and Miller, 1948), abnormal retention of both would be expected to occur in the same type of conditions, e.g. biliary obstruction and hepatocellular damage. This is, indeed,

the case. However, it would appear that the test dose of bromsulphthalein employed constitutes a greater functional burden on the excretory mechanism than does the amount of bilirubin formed in the course of normal degradation of haemoglobin. Consequently, abnormal bromsulphthalein occurs practically invariably in the presence of hyperbilirubinaemia, except, of course, that due to excess haemolysis. The bromsulphthalein test is, therefore, of no diagnostic value in patients with clinical jaundice, (except haemolytic jaundice) or those with an abnormally high serum bilirubin concentration. It should be noted, however, that bromsulphthalein can be measured in jaundiced serum by proper photoelectric colorimetric procedures and that the presence of jaundice or liver damage does not itself contra-indicate the use of this procedure (Gaebler, 1945). The bromsulphthalein test is particularly useful in the study of patients with hepatitis or biliary tract disease in whom the serum bilirubin concentrations are within normal limits.

Hepatic cell damage.

Abnormal retention of bromsulphthalein in the blood occurs almost invariably in acute hepatic disease, the degree of retention being roughly indicative of the

extent of hepatic functional impairment. Bromsulphthalein retention is one of the most sensitive tests for the recognition of hepatic-cell damage and can be used for screening purposes after exposure to hepatotoxic drugs (such as arsphenamine and carbon tetrachloride used therapeutically), or during epidemics of hepatitis, or for the evaluation of liver function during recovery from hepatitis (Barker, Capps and Allen, 1945). According to Havens (1948), it is the first test to show abnormal results in early viral hepatitis. In cirrhosis without jaundice significant retention is often found, permitting differentiation from other types of hepatomegaly. Zamcheck, Chalmers, White and Davidson, (1950) suggested the test should be used to differentiate massive gastro-intestinal haemorrhage caused by oesophageal varices in cirrhosis of liver from other sources of bleeding. In fatty livers without cirrhosis, bromsulphthalein retention is usually high.

In the presence of obstruction of the common bile duct, the degree of retention of bromsulphthalein increases progressively with increasing bilirubinaemia until all of the injected dye remains in the blood at the end of the test period. In the presence of ob-

struction, therefore, no information can be obtained by this method which cannot be obtained by means of the quantitative determination of serum bilirubin. After relief of the obstruction, dye retention, although diminishing, frequently persists for a variable period of time after the serum bilirubin has returned to normal. Cantarow and Trumper (1955) consider that this is due to the residual liver damage which is present in nearly all patients who have suffered from biliary obstruction for an extended period, especially that due to biliary calculi. Burnett, (1954) has noted that even in acute cholecystitis without jaundice, dye retention is often noted.

Other Conditions.

Abnormal bromsulphthalein retention occurs in conditions in which the hepatic blood flow is diminished, with or without liver cell damage. These include congestive cardiac failure (Felder, Mund and Parker, 1950), and in some cases of medical and traumatic shock, (Davidson, Lewis, Tagnon, Adams and Taylor, 1946). However, the hepatic blood flow is often essentially normal in shock and haemorrhage, in the absence of intrinsic liver damage, and the amount

of bromsulphthalein retention does not parallel the severity of shock or amount of blood loss. In such conditions, impaired liver damage may result from anoxaemia of liver cells due to vasoconstriction of the portal vein and hepatic artery branches, It may also explain the increase (usually transient in nature) in bromsulphthalein retention following operative procedures, particularly those involving the bile passages (Tagnon, Robbins and Nichols, 1948). A slight transient retention of bromsulphthalein may occur in the presence of fever in the absence of demonstrable liver damage (Hicks, Holt, Guerrant and Leavell, 1948). Bromsulphthalein retention may be elevated in thyrotoxicosis, malaria (Machella, 1947) diabetes mellitus (Pomeranze, 1951, 1952), pneumonia and other infectious diseases. Abnormal retention often occurs in the metastatic spread of carcinoma to the liver (Mendelsohn and Bodansky, 1952; Paulson and Wyler, 1942; Schneeberg, Likoff and Meranze, 1943). The reduced bromsulphthalein extraction in premature infants and its improvement with maturation have been related to the gradual development of the hepatic circulation (Obrinsky, Denley and Brauer, 1952). Determination of the bromsulphthalein content in the

serum several days after the administration of the test dose indicates that retention of the dye persists much longer in obstructive jaundice than in acute hepatitis and cirrhosis (Giges, 1951).

5. BROMSULPHTHALEIN TEST ON EXPERIMENTAL ANIMALS

The test has been performed on many different animals, especially cats, dogs and rats, and some of these have already been reviewed. Retention of the dye has been noted in dogs with biliary fistulae (Drill, Annegers, Snapp and Ivy, 1945) or with carbon tetrachloride intoxication (Gornall and Bardawill, 1952) and in rats with experimental fatty liver (Koch-Weser, Faber and Popper, 1951), bromobenzene intoxication (Koch-Weser, de la Huerga Yesinick, and Popper, 1953), ligated common bile ducts (Koch-Weser, Meyer, Yesinick and Popper, 1952) or nutritional liver damage (Koch-Weser, de la Huerga and Popper, 1953). The test can also be used in mice (Casals and Olitsky, 1946) and hens (Campbell, 1957).

6. BROMSULPHTHALEIN EXCRETION IN BILE

Studies of the curve of elimination of bromsulphthalein in the duodenal fluid (by duodenal intubation) with or without simultaneous estimations of

the dye in the blood, may give added information (Cantarow and Trumper, 1955; Wirts and Cantarow, 1942). Under normal conditions with the 2.0 mg. per Kg. dose of bromsulphthalein, dye appears in the bile within 15 minutes. In the first hour, 50 - 80 per cent of the quantity injected is excreted in the bile and 60 to 100 per cent in two hours. The curve of excretion reaching a maximum level at forty-five to seventy-five minutes and falling to a relatively low level at two hours. These findings suggest that the dye is rapidly removed from the blood and is subsequently excreted more slowly into the bile. All the bromsulphthalein cannot be recovered in the faeces, because some of it is destroyed by intestinal bacteria (Giges, Morse, Sharon and Wynn, 1953).

In some cases, especially with incipient biliary obstruction or slight hepatocellular degeneration this method is valuable since the biliary excretion is delayed in the absence of abnormal dye retention in the blood. Bromsulphthalein excretion can also be used to differentiate gall bladder bile from liver bile (Schlam, 1952). However, the usefulness of the procedure is limited by the fact that duodenal intubation is required and by certain technical difficulties.

7. HEPATIC CLEARANCE OR EXTRACTION OF BROMSULPHTHALEIN

Hepatic bromsulphthalein clearance represents the amount of blood cleared of bromsulphthalein by the liver in any unit of time; the hepatic extraction ratio is the amount of bromsulphthalein removed by the liver also in any unit of time. Both quantities, of course, depend upon the plasma volume. The clinical aspects are considered in this section and the mathematical interpretations are discussed in the following section.

Clearance can be calculated from data obtained from the peripheral blood with the help of mathematical formulae (Lavers, Cole, Keeton, Gephardt and Dyniewicz, 1949; Mendeloff, Kramer, Ingelfinger and Bradley, 1949; Goodman, 1952; Evans, 1953; Nadeau, 1954), and based on the assumption that bromsulphthalein is removed only by the liver and that the disappearance rate follows a simpler logarithmic curve. Since these assumptions may not necessarily be valid, especially in the presence of a pathological liver, the use of the term 'clearance' is possibly not justified. The bromsulphthalein extraction ratio requires examination of hepatic vein blood obtained by catheterisation (Ingelfinger, Bradley, Mendeloff, and Kramer, 1948; Sherlock, Bearn, Billing

and Paterson, 1950). Although theoretically ideal, it is too difficult for practical purposes.

Use of the technique of cardiac catheterisation has been made for the estimation of hepatic blood flow, since by venous catheterisation it is possible to obtain blood directly from the hepatic veins in the living subject (Bradley, Ingelfinger, Bradley and Curry, 1945). The blood flow is then determined on the basis of the Fick principle using bromsulphthalein given as a continuous infusion. According to this principle, the amount of bromsulphthalein removed per minute from the blood divided by the amount removed from each millilitre of blood passing through the organ equals the rate of blood flow. This requires knowledge of (a) the concentration or bromsulphthalein flowing into the liver, (b) its concentration in blood leaving the liver and (c) the total amount removed from the blood by the liver per unit of time. The first factor can be determined from the peripheral blood levels, the second factor determined in the hepatic venous blood and the third factor is the amount which has to be injected to maintain a constant blood level of bromsulphthalein. There are several errors in the application of the Fick principle with the use of bromsulphthalein. These are :- (i)

the peripheral bromsulphthalein concentration may not necessarily be the concentration entering the liver (probably only of minor significance), (ii) the blood obtained from one hepatic vein may not be representative of all, (iii) bromsulphthalein may be extracted by other organs (Selkurt, 1953) and may be returned to the blood from the intestine (Lorber and Shay, 1952). If, however, the bromsulphthalein level is kept between 1.0 and 2.0 mg. per 100 ml. this source of error is rather insignificant (Sherlock, Bearn, Billing and Patterson, 1950), (iv) some blood from the splanchnic area may by-pass the liver, but in the absence of large collateral blood vessels this amount is considered insignificant. For these reasons, terms 'estimated hepatic blood flow' or 'estimated splanchnic blood flow' as suggested respectively by Bradley et al (1945) and Sherlock et al (1950) should be used for the results obtained. Based on the estimation of the urea production, Myers (1947) has confirmed the validity of the basic principles of the determination of the estimated blood flows using the method of Bradley et al (1945). Results in animals, especially dogs, based primarily on stromuhr and catheterisation methods are similar to those in man (Casselman and Rappaport, 1954; Lipscomb

and Crandall, 1947; Grindlay, Herrick and Mann, 1941).

Various physiological factors influence the liver blood flow, the following are associated with a decreased blood flow :- vertical posture (Culbertson, Wilkins, Ingelfinger and Bradley, 1951), exercise (Bradley, 1949), conditions associated with falls in blood pressure such as fainting and general anaesthesia (Ginsburg and Grayson, 1954; Shackman, Graber and Melrose, 1953), these are all cases where there is a generalised splanchnic vasoconstriction. Fever increases the liver blood flow due to the increased peripheral blood flow (Bradley, 1951), and an elevation of blood pressure increases the liver blood flow (Ginsburg and Grayson, 1954). Pregnancy does not influence liver blood flow, despite an increase in the blood volume (Munnell and Taylor, 1947).

Bile acids, cinchopen, acetylcholine and hexamethonium increase the liver blood flow (Grodins, Osborne, Ivy and Goldman, 1941; Ginsburg and Grayson, 1954).

IX. BROMSULPHTHALEIN 'TESTS' AND THE USE OF THE DYE IN THE MEASUREMENT OF LIVER BLOOD FLOW

This section deals specifically with the various types of tests and application to the abnormal or diseased liver. The basis of the original test which was known at first as the 'bromsulphthalein retention test' and later simply as the 'bromsulphthalein test of liver function' was a comparison of the amount of dye remaining in or retained in the plasma at a certain time following the injection of the standard amount of dye, compared with the so-called normal retention at this time. A slightly more sophisticated use of bromsulphthalein in the assessment of liver function was based on the measurement of the actual rate at which bromsulphthalein was removed from the plasma by the liver. The rapidity with which bromsulphthalein disappeared from the plasma of a normal animal had impressed a number of workers and it seemed reasonable to them to use a deviation from a normal or standard rate of removal as an indication of liver dysfunction. In one such application of the bromsulphthalein test, the actual percentage of dye removed from the plasma in unit time was calculated and called the 'percentage disappearance rate'(P.D.R.) In another application, the logarithms of the concentrations of bromsulph-

thalein in several samples after the injection of bromsulphthalein were plotted against time and since the logarithms of the concentrations seemed to lie on a straight line the slope of this line was used as an indication of the rate at which bromsulphthalein was leaving the plasma. The slope was called by some 'the clearance co-efficient', others were able to estimate from it the actual bromsulphthalein 'clearance', i.e. the volume of plasma completely cleared of dye in unit time. A less 'quantitative' approach was the use of a graphical representation of the rate of decline of plasma bromsulphthalein concentration after a single intravenous injection. In this method, samples of blood were taken at intervals after the bromsulphthalein injection and concentrations of dye (or their logarithms) plotted against time. The shape and character of the curve so produced was used to detect abnormality of liver functions. It was sometimes combined with the measurement of P.D.R. but not always. These variations of the bromsulphthalein test demanded only a single injection of bromsulphthalein and the collection of a few samples of blood. Other variations called for a continuous infusion of bromsulphthalein. In one method, the quantity of dye

removed from the plasma and equilibrium concentration is measured and expressed as a 'clearance' of bromsulphthalein, i.e. the volume of plasma (at equilibrium concentration) which would contain the quantity of bromsulphthalein removed by the liver in the unit time. In another method a continuous intravenous infusion of bromsulphthalein is used to measure the maximum quantity of dye the liver can remove from plasma in unit time. Since this quantity depends on the ability of the liver to 'de-saturate' itself by excreting bromsuplhthalein into the bile, this type of test measuring the maximum secretory capacity of the liver was referred to as L_m. Finally bromsulphthalein by continuous infusion has been used to measure liver blood flow.

1. THE BROMSULPHTHALEIN RETENTION TEST.

The Bromsulphthalein Retention Test has been widely used in the assessment of liver disorder. It has been indeed the most popular of all the variants of the test mentioned above. The test seems to have been applied in the assessment of all known liver disorders and the literature on the subject is immense.

In no case of proved liver dysfunction has the bromsulphthalein retention test failed to indicate abnormality and the test is regarded by the majority of workers as a reliable and safe method of distinguishing the abnormal from the normal liver. The apparent simplicity of the test too has appealed to many and added to its popularity. The previous section dealing with the clinical experimental uses of bromsulphthalein has to some extent covered this clinical aspect, although it has only fringed on a portion of the vast literature which has accumulated on this subject. The rationale of this test has also been briefly mentioned in that section. There are three main requirements, firstly the injection intravenously of a known quantity of bromsulphthalein (as a 'single injection), secondly the estimation of the plasma concentration of bromsulphthalein in one blood sample ('single sample') and thirdly the assumption that the plasma volume of the subject is related to body weight in the proportion of 50 mls. of plasma per Kg. body weight. The quantity of dye used has varied and the time of blood sampling has also varied but following the work of Mateer, Baltz, Marion and McMillan (1943) the dose has generally been 5 mg./Kg./body weight and

the single sampling time 45 minutes. These workers investigated healthy adult subjects 25 - 30 years of age. Blood samples were taken every 5 minutes for 45 - 60 minutes after the injection of a 5 mg./Kg./ body weight dose of bromsulphthalein. These workers recorded that in 30 minutes all the bromsulphthalein had been completely removed from the plasma in 73% of their control group. At 35 minutes, 86% of the controls had been completely cleared and at 45 minutes all had plasma free of bromsulphthalein. They concluded 45 minutes from zero was a useful standard of time for use after a 5 mg./Kg. dose of bromsulphthalein since at this time any retention at all would indicate liver dysfunction. They suggested that serial sampling was not routinely necessary and recommended the taking of only two samples, one at 45 minutes and one at 60 minutes from the time of injection. Mateer et al (1943) completed their investigation by showing that the bromsulphthalein test as they performed it correlated well with other tests of liver function, particularly the cephalin-cholesterol test. As a result of this and other similar works the practice of taking blood at 45 minutes became almost standard and significant retention, i.e. more than five per cent, was

found to be commonly associated with this disorder. Giges (1951) who performed the test on 35 patients with known liver disease found in all cases retention at 45 minutes was over 20 per cent.

Despite the apparent success of the bromsulphthalein test indicating liver damage when it can be shown by independent methods to exist, it is clear that the test can never be wholly satisfactory while it is based on two assumptions which are open to question. The more important of these two assumptions concerns the plasma volume. It is unlikely that all subjects will have a plasma volume which is predictable on the simple body weight basis of 50 mls./Kg. It therefore follows that a subject with both an abnormal liver and a plasma volume greater than the normal might well show the normal retention of bromsulphthalein since the concentration of dye will be low at 45 minutes, not because the liver is normal but by virtue of the diluting effect of a large plasma volume. The second and less important assumption concerns the hypothetical plasma bromsulphthalein concentration at zero time, i.e. the moment the injection is given. For the purposes of the test, mixing of the dye in a whole plasma volume is assumed to be instantaneous , whereas of course this is impossible.

It is almost certain that the removal of some of the dye by the liver precedes the full diffusion of the dye within the plasma. On these two counts, therefore, the Bromsulphthalein Retention Test is open to serious criticism.

2. THE PERCENTAGE DISAPPEARANCE RATE (P.D.R.)

Meaurement of the rate at which bromsulphthalein leaves the plasma after a single injection was suggested by Ingelfinger and his associates as a possible method of assessing liver function. (Ingelfinger 1946; Ingelfinger, Bradley, Mendeloff and Kramer 1948; Mendeloff, Kramer, Ingelfinger and Bradley, 1949). Ingelfinger believed that after bromsulphthalein 150 mgs. per square meter surface area is injected intravenously its disappearance rate from the blood of normal subjects is such that a constant percentage of the dye present in the blood is removed per minute - between 10 and 15% per minute (Ingelfinger 1946), He had observed what seemed to him to be a straight-line relationship between time (minutes) and the logarithms of the plasma bromsulphthalein concentrations measured at 6, 12, 20 and 30 minutes after injection of the dye. (He accepted a rectilinear relationship if a straight line could be

fitted to the plotted values in such a way that none of the points deviated from the line by more than 0.2 mgs./100 mls. This straight line relationship was taken to mean that the plasma bromsulphthalein concentration decreased in an exponential fashion and the percentage disappearance rate which was described was the exponential function governing the rate of decrement of plasma bromsulphthalein concentration. Ingelfinger calculated the P.D.R. as follows :-

1. If the plasma bromsulphthalein concentration at time $t_1 = C_1$, the plasma bromsulphthalein concentration at time $t_2 = C_2$

the d = exponential function governing the rate of decline, i.e. $d = e^{-k}$, where k is the actual slope of the plotted line.

Since the decline is exponential, then,

$$C_2 = C_1 \ (d)^{t_2 - t_1}$$

$$\therefore \text{and} \quad \log \frac{(C_2)}{(C_1)} = (t_2 - t_1) \log d$$

$$\text{Percentage Disappearance Rate (P.D.R.)} = \frac{(1 - d) \times 100}{}$$

In 72 tests which Ingelfinger et al (1948) performed on 55 healthy adults, 66 showed the desired

straight line relationship between time and the logarithms of the plasma bromsulphthalein concentrations. The mean P.D.R. was 13.6 per cent per minute (the S.D. = 1.9), that is to say 15.6 of the quantity of dye present in the plasma at any time was removed by the liver in one minute. In a further group of 107 tests on 103 hospital patients who suffered from a variety of complaints, but were apparently free from liver disease, Ingelfinger found that in 76 tests (71%) a straight line relationship was obtained between time and the logarithms of the plasma bromsulphthalein concentrations. The P.D.R. in these cases averaged 12.8 $\pm$ 2.45% per minute. In the remaining 31 tests a straight line relationship was not demonstrated, - six of them were called 'irregular' and 25 showed a 'saturation'. By 'saturation' Ingelfinger meant that the slope of the plotted curve was not constant but decreased with time. A different, less steep slope, was usually evident between 20 and 30 minutes after the injection of bromsulphthalein when the concentration of dye was low (about 0.5 mgs./100 mls.) Ingelfinger believed therefore that in 'most healthy subjects and in the majority of patients without liver disease' a constant proportion of bromsulphthalein

disappeared from the blood per unit time. This constant proportion - the P.D.R. had, in the series quoted, a normal range of 10 - 16% per minute. However, it is clear from Ingelfinger's writing that he was not completely satisfied with this interpretation for he wrote (Ingelfinger et al, 1948) :-

> In the case of bromsulphthalein, the constant P.D.R. is usually maintained for the first 30 minutes following injection of the dye, but in some cases if the dye content of the serum is extremely low, toward the end of this time interval P.D.R. may fluctuate considerably. Since a deviation of 0.2 mgs./100 mls. is allowed by definition, however, changes in P.D.R. of this type are disregarded, a procedure which can be defended since it applies only to low levels of bromsulphthalein when more than 95% of the injected dose has disappeared. A curve which according to definition manifests 'saturation' indicates that P.D.R. is increasing while significant amounts of bromsulphthalein remain in the plasma. A curve of this type was found in roughly one out of four hospital patients even though no clinical evndence of hepatic dysfunction

was apparent. The disappearance of bromsulphthalein after the 30 minute interval was not studied but it is possible that a decrease in P.D.R. might be found not infrequently if accurate analysis of bromsulphthalein were feasible at very low concentrations".

It is clear then that the conception of a constant percentage disappearance rate of bromsulphthalein can only be accepted for a part of the time during which bromsulphthalein in plasma is exposed to the removal mechanism of the liver, and such acceptance even cannot be complete for there are two main defects in Ingelfinger's method: first, four plotted points are not sufficient to define a straight line with accuracy (the bromsulphthalein concentrations of 6, 12, 20 and 30 minutes were used), and secondly, the acceptance of a deviation of 0.2 mgs./100 mls. in 'low' concentrations of bromsulphthalein makes it virtually impossible to accept as 'true' any concentration below, say, 0.8 mgs./100 mls. and this concentration was reached in more than half of the cases quoted by Ingelfinger et al (1948) in less than 20 minutes from the time that the injection of bromsulph-

thalein was given. Despite these limitations, the measurement of P.D.R. was carried out on a group of patients with known hepato-biliary disorders. It was shown (Ingelfinger et al, 1948) that patients with cirrhosis of the liver showed a reduction in P.D.R. (range 1.9% to 8.2% per minute in 46 tests) whereas patients with 'fatty infiltration of the liver' showed an increase in P.D.R. (18.5 to 22.5% per minute in five tests). The ability of the fatty liver to remove bromsulphthalein from plasma more rapidly than a normal liver was not awarded an explanatory note in Ingelfinger's report.

3. THE 'CLEARANCE CO-EFFICIENT' OF BROMSULPHTHALEIN

The straight-line relationship between time (minutes) and the logarithm of plasma bromsulphthalein concentration alleged to exist by Ingelfinger has been the basis of another attempt to express quantitatively the ability of the liver to remove injected bromsulphthalein from the plasma. In this case the slope of the line was used as an indicator of liver normality and the slope was called the 'clearance co-efficient'.

If it can be accepted for the purposes of discussion only that the disappearance of bromsulph-

thalein from the plasma after a single injection follows a single exponential pattern, then,

$$CN_{t_2} = CN_{t_1} e^{-k(t_2 - t_1)}$$

where CN_{t_2} = the bromsulphthalein concentration at any time $_2$

CN_{t_1} = the bromsulphthalein concentration at time t_1, before t_2

k = the slope of the plotted points.

Now CN_{t_1} and CN_{t_2} and t_1 and t_2 are variables which can be measured and so the values of 'k' (the slope) can be calculated.

This, in essence, was the method used by Goodman (1952) and Lavers, Cole, Keeton, Separdt and Dyniewicz (1949) to calculate their 'clearance co-efficient'. They calculated this 'co-efficient' in both healthy adults and in patients with known liver disease. Blood samples were taken at 5, 10, 15, 20, 30 and 45 minutes after the injection and plasma bromsulphthalein concentrations were plotted against time on semi-logarithm paper. The slope of the resulting straight-line was calculated as described

above. Lavers et al (1949) found the average 'clearance co-efficient' in normal healthy adults to be 0.082 and the lower limit of 90% of their controls to be 0.037. Goodman (1952) whose control subjects were all adult males found the range of normal to be 0.057 to 0.084 with a mean of 0.067. There is, therefore, reasonable agreement between their results.

It is interesting, however, to compare these figures with those obtained by Ingelfinger. This is possible, since the P.D.R. can be calculated from a knowledge of the slope alone as follows :-

$$P.D.R. = 100 (1 - d)$$

and $d = e^{-k}$, where k is the slope of the line joining the plotted points

therefore if $k = 0.082$ (Lavers et al 1949) the

$P.D.R. = 100 (1 - e^{-.082}) = 8\%$

and $k = 0.067$ (Goodman, 1952) the P.D.R. =

$100 (1 - e^{-.067}) = 7\%$

These values for P.D.R. are less than the normal values (10 - 16%) found by Ingelfinger (1948). The reason for the difference is not clear, for although the subject in the series of Goodman, Lavers et al were all hospital patients (said to be free from liver disease) their mean P.D.R. was less than that of Ingelfinger's

hospital group (12.8%). The range of P.D.R. in Ingelfinger's hospital group was 8.5 to 23% and it may be that it included more cases of undiagnosed liver disorder than Ingelfinger imagined. Conversely, all the subjects in the 'clearance co-efficient' group may have had latent liver disease but this is unlikely in view of the close agreement of values in the two separate observations. One further point of great interest arises out of the work of Goodman (1952) and Lavers et al (1949). It was noted in both reports that the straight line relationship between time and the logarithm plasma concentrations did not always hold and it was found that in some cases the slope of the line began to change at about 20 minutes. Goodman thought the change in slope might reflect re-absorption of bromsulphthalein from the intestine. Laver et al suggested that it might be related to the rate of excretion of bromsulphthalein into the bile but neither regarded the phenomenon with any great seriousness and it was assumed that its occurrence did not in any way alter their interpretation of the observed 'facts'.

BROMSULPHTHALEIN CLEARANCE (SINGLE INJECTION METHOD) 'FRACTIONAL CLEARANCE'

Lewis (1948, 1950) has shown how a 'Fractional Clearance' of bromsulphthalein may be derived from the changes of plasma concentration in bromsulphthalein following intravenous administration of the dye as a single injection. The work was based mainly on the assumption that there exists a straight line relationship between time and the logarithm of the plasma concentration, and Lewis was careful to point out that this was no more than an assumption which made it possible for him to derive 'fractional clearance'. The term 'fractional clearance' was defined as that fraction of the plasma volume which was cleared of bromsulphthalein in unit time. Lewis preferred to express 'clearance' as a ratio rather than in precise volumes, for he appreciated the general unsatisfactoriness of efforts to determine the plasma volume in a reliable way. His method of deriving the 'fractional clearance' can be described briefly as follows :- A single injection of bromsulphthalein (5 mg./Kg. body weight) is given intravenously and blood samples are then taken at 5 or 10 minute intervals for 40 to 60 minutes. The times are recorded and

the plasma bromsulphthalein concentrations of the blood samples are estimated.

The derivation of the 'fractional clearance' is as follows :-

Let P = the initial plasma concentration at time t_1

P_1 = a final plasma concentration of bromsulphthalein (greater than zero) at time t_2

P_m = the mean bromsulphthalein concentration during the time interval between P and P_1

C = the volume of plasma cleared during the time interval $(t_2 - t_1)$

V = the plasma volume

Q = the initial quantity of bromsulphthalein present in the plasma at time t_1

Q_1 = final quantity of bromsulphthalein present in the plasma at time t_2

$t_2 - t_1$ = the clearance period.

The quantity of bromsulphthalein removed during the clearance period = $C \times P_m$

Then $Q - CP_m = Q_1$ dividing by V

We get $\frac{Q}{V} - \frac{CP}{V}_m = \frac{Q_1}{V}$

but $\frac{Q}{V} = P$ and $\frac{Q_1}{V} = P_1$

$\therefore P - \frac{CP}{V}_m = P_1$

that is to say $P - P_1 = \frac{CP}{V} m$

and $\frac{P - P_1}{P_m} = \frac{C}{V}$

Now it is assumed that the declining concentrations of bromsulphthalein fall along a logarithmic curve and so P_m is approximately equal to the geometrical mean of P and P_1,

i.e. $P_m = \sqrt{P \times P_1}$

$\therefore \frac{P - P_1}{\sqrt{P \times P_1}} = \frac{C}{V}$ = 'Fractional Clearance' for interval $t_2 - t_1$

This fraction, $\frac{C}{V}$ can be calculated from two plasma bromsulphthalein concentrations and represents the fractional clearance for the interval between samples P and P_1.

Lewis commonly employed a time interval of 10 - 15 minutes but by suitable multiplication expressed his 'fractional clearance' 'per hour'. This implied that the relationship between time and the logarithm plasma bromsulphthalein concentration remained rectilinear for the 60 minutes. It has been appointed above that this is not necessarily the case. The 'fractional clearance' per hour for normal humans was found to be 11 and for dogs 12.8.

5. GRAPHICAL REPRESENTATION OF THE TIME COURSE OF BROMSULPHTHALEIN IN PLASMA AFTER INJECTION

Macdonald (1939) suggested that the shape of the curve obtained when plasma bromsulphthalein concentration was plotted against time could be used in distinguishing normal from abnormal liver function in humans. He surveyed a very large series of hospital patients, some with normal livers and some with suspected liver disease. He used the standard dose of bromsulphthalein (5 mg./Kg. body weight) and took blood samples fairly frequently (5 - 10 minute intervals) for 45 - 60 minutes after injection of the dye. The plasma bromsulphthalein concentration (not the logarithms) were plotted against time. Macdonald illustrated his result with innumerable graphs and though he was able to say there was some gross difference in shape between the 'normal' and 'abnormal' curve, his work was in no sense quantitative and he suggested no real criteria by which the abnormal could be distinguished from the normal.

Obrinsky et al (1952) adopted a similar method in an investigation of liver function in the premature infant and neonate. They accepted as fact that, following a single intravenous injection of bromsulph-

thalein a constant proportion of the dye was removed from the blood per unit time and therefore they used a plot of plasma concentration as against time. Their assessment of liver function was in fact based on the slope of the line obtained in such a plot but they did not measure the slope. They preferred rather to describe it as 'steep' or 'less steep' and they demonstrated that normal livers were associated with 'steep' slopes and abnormal livers were associated with 'less steep' slopes, or lines which showed changes in the slope. They were prepared to accept, however, that in some cases normal liver function could be associated with the departure from true logarithmic decline in plasma bromsulphthalein concentration.

6. TRUE CLEARANCE OF BROMSULPHTHALEIN - (CONTINUOUS INJECTION METHOD).

By the 'true' clearance of bromsulphthalein is meant the actual volume of plasma totally cleared of bromsulphthalein in unit times. To measure this Vershure (1952) used a method the same in essence as that one used to measure renal clearance of various substances. Bromsulphthalein was given by continuous

intravenous infusion (in human subjects) until the plasma concentration remained steady. This concentration was called the 'Equilibrium Concentration". During equilibrium conditions the amount of bromsulphthalein leaving plasma per minute via the liver was considered to be equal to the quantity of bromsulphthalein being infused per minute. Therefore, the clearance can be calculated by dividing the quantity removed per minute by the plasma bromsulphthalein concentration at equilibrium.

Clearance $\therefore$ =

$$\frac{\text{Infusion rate mg./min.}}{\text{Plasma bromsulphthalein concentration mg./100 ml.}} \times 100$$

Versheure (1952) made 64 clearance measurements on 40 normal adults. The continuous infusion of bromsulphthalein was at the rate of 2, 5 or 10 mg. per minute was proceeded by a loading dose of between 25 and 70 mgs. bromsulphthalein. Blood samples were taken at 5, 7, 9, 11, 12, 13 and 15 minutes from the beginning of the infusion. Vershoure claimed that 'equilibrium' was reached in 5 minutes but it is very difficult to understand how this could be so. Clearly,

with the relatively small loading dose used at the lower rate of infusion (205 mg. per minute) the change in plasma bromsulphthalein concentration in five minutes might have been slight and apparently acceptable as an 'equilibrium' level, but unless the concentrations of subsequent samples were very close to those of the first two samples it would be impossible to know whether plasma levels were steady or changing. From Vershure's report it is not possible to picture the rate of change of plasma bromsulphthalein concentrations before the establishment of 'equilibriums'. He only reported plasma levels 'at equilibrium'. In general he found the clearances with plasma levels above 1 mg./100 mls. were considerable lower than those with lower plasma levels. The clearance could be grouped in fact according to the plasma levels as follows :-

Group	Plasma level (Mg/100 ml.)	Clearance (ml/min)	No. of cases
1	0.0 - 0.5	2,327	14
2	0.5 - 1.0	795	13
3	1.0 - 1.5	789	14
4	above 1.5	533	7

Vershoure accepted group 2 and 3 as being probably the most reliable. He suggested that the lower clearance in group 4 was possibly due 'to decreasing percentage extraction of the dye in the liver'. Group 1 he also rejected without making his clear reason for doing so. It is interesting to note that the rates in Group 1 were either 2 or 5 mg./min., and that the rate in Groups 2, 3 and 4 was (with one exception in Group 2) 10 mg./min. That is to say, in Group 1, for instance, the liver was able to remove approximately 40% of the quantity of bromsulphthalein in the plasma in one minute; in Group 3 only 20% per minute was removed. This difference is inexplicable in terms of the accepted simple idea of bromsulphthalein removal from plasma which was favoured by Ingelfinger, Lewis, Goodman and Lavers. Indeed, on the basis of this simple idea, it is difficult to understand how a continuous infusion of bromsulphthalein could ever result in 'equilibrium', and certainly not in five minutes.

7. THE MAXIMUM EXCRETORY CAPACITY OF THE LIVER.

If it can be accepted that, during the continuous intravenous infusion of bromsulphthalein

equality between the amount of bromsulphthalein presented to the liver and the amount removed by the liver in unit time results in an unchanging plasma level or bromsulphthalein level, it follows that the presentation in unit time of an amount greater than that which could be removed in the same time would result in continually rising plasma bromsulphthalein concentration. If the difference between the rate of presentation of bromsulphthalein (infusion rate, mg./min.) and the removal rate (= biliary excretion rate, mg./min.) were constant, then the resulting rise in plasma level would be linear with regard to time, since a constant amount of bromsulphthalein (infusion rate minus excretion rate) would be added to the plasma in unit time. Therefore, when administration of bromsulphthalein at a constant rate results in a concentration which increases linearly with time, the presumption is that the removal or excretory rate of bromsulphthalein by the liver is also constant, and represents the greatest quantity of bromsulphthalein which the liver can excrete in unit time. If the infusion rate and the rate of plasma dye increase are known, the rate of excretion by the liver can be simply calculated.

Thus, if Q = infusion rate of bromsulphthalein mg./min.

and P_1 = rate of plasma dye content increase, mg./min.

and L_m = maximum rate of excretion by the liver, mg./min.

$$\text{then } Q - L_m = P_1$$

$$\text{or } L_m = Q - P_1$$

The maximum rate of bromsulphthalein excretion by the liver (L_m) has been called the Maximum Excretory capacity of the liver (for bromsulphthalein) by Mason, Hawley and Smith (1948).

Mason et al (1948) measured the rate in the normal dog and found it to be about 0.44 mg. bromsulphthalein/Kg. or 7.5 - 10.3 mg./min./M^2. They used continuous intravenous infusions in the region of 0.45 mg./Kg./min. preceded by a 'priming' or 'loading' dose of bromsulphthalein (15 - 30 mg./Kg.) and achieved steady plasma levels of bromsulphthalein 60 minutes from the beginning of the infusion. The infusion rate was then increased until the plasma bromsulphthalein concentration showed a linear rise with time. The infusion rate was recorded, and two plasma samples were taken 5 - 10 minutes apart. With this information, together with a measure of the rate of renal loss of

bromsulphthalein and an estimate of the plasma volume, I_m was calculated from the formula :-

$$I_m = (Q - \text{urine loss/min.}) - \frac{V(P - P_1)}{T}$$

where Q = bromsulphthalein infusion rate/mg./minute

V = plasma volume (estimated from the Haematocrit and blood volume as a percentage of body weight)

P = bromsulphthalein concentration of the first plasma sample

P_1 = bromsulphthalein concentration of the second sample

T = time (minutes) between the two samples, P and P_1

Versheure(1952) applied the same formula and a similar method to the measurement of the Maximum Excretory capacity in man. In 29 normal men, I_m was found to be 18.3 mg./min. (Range 15 - 21 mg./min. the standard deviation being 1.6). It is not clear from Versheure's report however, that the methods he used were entirely adequate. Using a loading dose of 62.5 - 125 mg. bromsulphthalein and an infusion rate of 25 mg./min., Versheure claimed to have achieved a

linear rise in plasma bromsulphthalein concentration within 15 minutes and none of his observations seem to have been made over a longer periodtthan this. It seems unlikely that a true appreciation of the nature of the rise in plasma bromsulphthalein concentration would be possible in so short a time.

The accuracy of the method also depends on the accuracy assigned to the plasma volume and, while the figures used by Versheure (50 ml. plasma per Kg. body weight) may be sufficiently accurate in the case of normal humans, it does not follow that the figure can be applied in abnormal states. Versheure's report on the range of L_m (3 - 17.6 mg./min) in 20 patients with known liver disease can, therefore, only be accepted with reserve.

8. THE MEASUREMENT OF LIVER BLOOD FLOW

The flow of blood through the liver has been studied in a number of different ways. Since one of these methods involves the use of bromsulphthalein given as a continuous infusion, it is necessary to briefly review these methods before fully with the technique using bromsulphthalein.

(a) Measurement of blood flow in the main vessels of the liver.

Burton-Opitz (1910, 1911) using a mechanical stromuhr reported measurements of the blood flow in the portal vein and hepatic artery. Using anaesthetised dogs, he found the portal blood flow to be 84 ml./100 Gm. liver, and the hepatic artery flow to be 143 ml./min. per whole liver. Blalock and Mason (1936) measured the portal blood flow in conscious dogs and their results (82 ml./ 100 Gm. liver) are in substantial agreement with those of Burton-Opitz. Using thermostromuhrs in the portal vein, hepatic artery and thoracic vena cava, Grindley, Herrick and Mann (1941) found great variation in the rate of blood flow through the liver and rejected the idea that there was a correlation between the liver blood flow and the size of the liver. They found the hepatic artery flow to be 44.6 - 163 ml./ minute per whole liver in anaesthetised dogs. They noted also in two dogs the hepatic artery flow was greater than portal vein flow. They measured both in-flow and out-flow from the liver and were able to record instances in which the in-flow exceeded the out-flow and vice-versa. They suggested that the liver could store blood.

(b) Measurements of 'tissue perfusion' using the 'thermal conductivity' methods.

Grayson (1951a, 1951b, 1952) measured the local tissue perfusion in the liver using a heated thermocouple. This method yielded values, not for total liver blood flow, but for local fluid movement. One heated thermocouple recorded the fluid movement in a collar of tissue 0.5 cm. long and 0.3 cm. in diameter. Grayson claimed that thermal conductivity as measured by him, was approximately a linear function of blood flow, and that the excess thermal conductivity of living as opposed to dead tissue could be used quantitatively in its measurement. Although Grayson's method was not able to measure total blood flow, but only flow changes in circumscribed volumes of liver tissue, the reactions of the liver to haemorrhage, adrenalin, noradrenalin, acetylcholine and nerve stimulations were shown to be the same in all parts and in each lobe. Grayson found no evidence to support the occurrence of large spontaneous variations in liver blood flow, and he maintained that, in the absence of specific stimuli, blood flow was remarkably constant.

(c) Measurement of 'distributed' flow.

(i) Fractionation of the cardiac output.

Sapirstein (1956, 1958) has used a method of fractionating the cardiac output based on the assumption that intravenously injected K^{42} would be distributed initially to the various body tissues in proportion to the fraction of the cardiac output received by each tissue. It was assumed in these studies that there was sufficient time lag between delivery of the isotope to the tissue and its subsequent washout from the tissue, to permit collection of tissue samples which contained essentially all of the initial activity and no recirculated activity. The method provided also an estimate of hepatic artery and portal vein contributions to hepatic blood flow, since the K^{42} in the liver was assumed to give the hepatic artery contribution while the isotope content in the remaining splanchnic viscera gave the portal vein contribution.

In general, the results indicated that the hepatic in-flow was about one-third arterial and two-thirds portal and was therefore in agreement with the findings of Burton-Opitz (1910, 1911) but not with those of Blalock and Mason (1936) who considered the

ratio of portal vein/hepatic artery flow to be about 5 : 1. The figures given by Sapirstein are as follows :-

Total liver blood flow, 1.2 ml/min/Gm. of liver, i.e. 30% of the cardiac output.

Hepatic artery flow, 0.4 ml/min/Gm. of liver, i.e. 10% of the cardiac output

Portal vein flow, 0.8 ml/min/Gm. of liver, i.e. 20% of the cardiac output.

(ii) Fractionation of the extra splanchnic blood volume.

Dobson and Jones (1952) used radioactive colloidal chromic phosphate for the estimation of hepatic blood flow as a fractionation of the extra-splanchnic blood volume. They showed that when a suitable colloid was injected intravenously it disappeared from the blood-stream at a very rapid exponential rate. The polygonal cells of the liver and the spleen are the cells mainly concerned in the removal of such colloids from the blood. The equation which governs the disappearance from the blood of at least 90% of the injected material is as follows :-

$$C_t = C_o\, e^{-kt}$$

Where C_t is the concentration of colloid in the blood at time t,

C_o is the concentration at zero time

e is the base of the natural logarithms

and k is the disappearance constant.

The constant, k, represents the fraction of the colloid present in the peripheral circulation which disappears in unit time.

The liver blood flow can be calculated by multiplying the disappearance rate constant by the extra-splanchnic blood volume (Dobson, Warner, Finney and Adamson, 1956). It is important to note that the method is valid only if two assumptions can be made :- (i) the colloid must not be removed by extra-splanchnic tissues and (ii) the liver must be so highly efficient in the extraction of the colloid that hepatic vein blood carries no significant amount of the colloid back to the general circulation. Later work by Dobson (1958) has shown that these assumptions hold 'fairly well' in normal animals.

(d) The measurement of total blood flow

A widely used method for the determination of total blood flow across the liver is based on an application of the Fick principle. The concentration of

a substance which the liver removes from, or adds to the blood passing through it is measured in both the in-flow and out-flow vessels of the liver; this concentration difference is then divided into the amount of the substance the liver produces or removes in unit time, to give the hepatic blood flow in volumes/unit/time. Most commonly, a substance has been used which is removed from the blood by the liver. This substance is infused into a peripheral vein at a constant rate. When the substance reaches concentration equilibrium in the arterial blood, its delivery to the liver (hepatic blood flow x arterial concentration) will exceed the rate at which the substance leaves the liver in the hepatic vein (hepatic blood flow x hepatic venous concentration) by the rate of excretion (or removal) of the substance by the liver (Sapirstein 1958).

This relationship can be written as follows :-

$$HBF = \frac{Q}{A - HV}$$

where HBF = the hepatic blood flow in ml/min.

Q = the amount of substance excreted by the liver mg/min.

A = the arterial blood concentration of the substance mg/ml.

HV = the hepatic venous blood concentration of the substance in mg/ml.

In this expression both A and HV can be measured directly but Q cannot be. It is, however, assumed that at equilibrium concentration Q is equal to the quantity of the substance being infused per minute. Since the infusion rate (I) can be measured, it can be substituted for Q in the above expression. To indicate that this assumption has been made the hepatic blood flow is usually referred to as the 'estimated' hepatic blood flow (EHBF) so that the expression becomes :-

$$EHBF = \frac{I}{A - HV}$$ (where I = the infusion rate mg/minute).

Before a substance can be used to estimate hepatic blood flow in this way it must, clearly satisfy certain criteria. It must be a substance which is confined to the plasma volume; it must be removed from the blood stream solely by the liver; it must not alter or damage the function of the liver in any way and it must not be the cause of alteration in any other function of the body. Finally, it must be possible to estimate the concentration of the substance in the blood. No substance which completely

matches these criteria has been found. Two substances which meet most, but not all, of these conditions are bromsulphthalein and tetraiodotetrachlofluorescein (Rose Bengal). These two substances have been extensively used in the measurement of hepatic blood flow.

Smyth, Heineman and Bradley, (1953) using bromsulphthalein estimated the hepatic blood flow to be 30 ml/Kg. body weight/minute. Using the same method, Werner and Horvath (1952) had previously given a value of 42 ml./Kg./minute and also in 1952, a value of 49 ml./Kg./min. was given by Pratt, Burdick and Holmes. Sapirstein and his co-workers (Sapirstein, Ezrow and Simpson, 1954; Sapirstein and Simpson, 1955) noted the variability in these (and other) values when bromsulphthalein was used to measure hepatic blood flow, and suggested that Rose Bengal was a more suitable substance. They believed, for instance, that Rose Bengal was confined to the plasma volume whereas bromsulphthalein had not been shown too conclusively to be so confined. In a clear exposition of the whole problem of measuring hepatic blood flow Sapirstein (1958) reported his results in the dog with Rose Bengal and in addition was able to

offer an explanation for the variability of results obtained by other workers using bromsulphthalein. He reported that, although Rose Bengal is a more suitable substance for measuring hepatic blood flow, its use alone will not exclude considerable error. He found the most important source of error to be the assumption that the composition of mixed hepatic venous blood was identical with the composition of a sample obtained by catheter from one hepatic vein. Sapirstein believed that the sampling catheter in the hepatic vein caused a partial obstruction. The obstruction resulted in an increased resistance to the in-flow of portal venous blood to the area drained by that hepatic vein. The resistance offered to the hepatic arterial supply would be insignificantly small, and hence the catheter lying in the hepatic vein would receive blood primarily of arterial origin, and a substance reaching the liver via the portal vein would not appear in representative concentration in that particular vein. To obtain a truly representative sample of mixed venous blood, Sapirstein used dogs in which he produced a common hepatic vein. He did this by cutting and tying the vena cava between the renal veins and the

diaphragm after a unilateral nephrectomy. The superior stump of the inferior vena cava was then converted into a common hepatic vein. Using this preparation (and Rose Bengal) Sapirstein (1958) found hepatic blood flow to lie in the range 50 - 60 ml./Kg./min., that is about 30% of the cardiac output. This value of 50 ml./Kg./min. is in good agreement with the results found using the methods of fractionation of the cardiac output and fractionation of the extra-splanchnic blood volume.

In man, measurement of the hepatic blood flow has been carried out using bromsulphthalein and hepatic vein catheterisation. Bradley (1950) gives values which range from 940 - 1860 ml./min. for a series of 19 estimations of 5 'normal' adults. There was considerable variation in his results and his explanation of this variation, although it involves the placing of a catheter in the hepatic vein was not the same as that given above by Sapirstein. Bradley suggested that the regurgitation of blood from the vena cava into the mouth of the hepatic vein and thence into the sampling catheter would explain the variability of his results.

It would seem, whichever explanation is the

true one, this method as it is usually carried out, cannot be accepted as providing more than an approximation to total liver blood flow.

An alternative method for obtaining samples of hepatic venous blood has been described by Shoemaker, Walker, Van Italie and Moore (1959a). This method avoids the passage of a large cardiac-type catheter into a hepatic vein. Through an abdominal approach a very fine polythene catheter is stitched into the left common hepatic vein and the free end then brought out through a stab incision in the left flank and sutured to the skin. Using this type of preparation, Shoemaker et al (1959b) were able to measure liver blood flow in the unanaesthetised animal. In 10 experiments on 9 dogs, they found liver blood flow to be 40 - 3 ml./Kg./min. This figure is lower than that given by Sapirstein (1958) using the artificially produced common hepatic vein of anaesthetised dogs and may indicate that, in general, the bromsulphthalein method using a conventional hepatic vein catheter, tends to over-estimate true hepatic blood flow.

Sherlock, Bearn, Billing and Patterson, 1950,

reported the measurements in 49 normal human adults using bromsulphthalein and their work reveals further complexities of this method when used for estimating hepatic blood flow. A priming dose of 150 mg. of bromsulphthalein was given followed by a continuous infusion of bromsulphthalein of 5, 6 or 7 mg./min. Plasma levels were observed for 2 - 3 hours, blood samples being taken at frequent intervals (5 - 20 minutes). It was found that the results could be divided into two groups depending on whether the plasma bromsulphthalein concentration was greater or less than 1 mg./100 ml. The mean infusion rate of bromsulphthalein in both groups was similar. In the first group of 32 subjects the peripheral bromsulphthalein concentration was greater than 1 mg./100 ml. and the mean liver blood flow was 836 $\pm$ 31 ml/min/M^2; the second group of 17 subjects had a plasma concentration below 1 mg./100 ml. and the mean liver blood flow was 1,530 $\pm$ 126 ml./min/M^2.

The apparent effect of peripheral plasma bromsulphthalein concentration on the estimation of liver blood flow is not unlike its effect on the estimation of the clearance by the method of Versheure (1952) referred to above. Low plasma levels seem to be

associated with the high clearance values and hence high blood values. In other words, at low plasma bromsulphthalein concentrations (below 1 mg./100 ml.) the proportion removed by the liver seems to be less than the proportion removed at high plasma concentrations. The relationship between percentage extraction of the dye and the plasma concentration is not readily explicable and is undoubtedly complex. It would seem that certain factors, other than simple 'hepatic removal' are involved and that they are, proportionately, more significant at low than high concentrations.

There is, however, one disturbing statement in the report by Sherlock et al (1950), namely :- "In 22 subjects the peripheral bromsulphthalein level showed a slow consistent rise during the 2 - 3 hour period, despite constant infusion". From the results given it is impossible to determine whether the observed rise in plasma bromsulphthalein concentration was linear in regard to time, but the use of the word 'consistent' to describe the rise seems to exclude any other interpretation. If a linear rise in plasma bromsulphthalein concentration did occur then it means that the infusion rate was

greater than the maximum bromsulphthalein excretory capacity for the liver of these subjects (the presumed L_m being 5 - 7 mg./min.), which is much lower than the figure given by Versheure (18.3 mg./minute) - range 15 - 21 mg./min. On the other hand, a rising plasma bromsulphthalein concentration could be due to a slowly rising rate of infusion. In this case, the rise in plasma concentration, although not strictly linear, might loosely be called 'slow and consistent'. In either case, it would seem unwarrantable to use the rate of infusion of bromsulphthalein as the rate of excretion of this substance (which is necessary if the Fick principle is to be applied) in the estimation of hepatic blood flow.

The estimations of liver blood flow are given by 24 different groups of workers in Table 1 and from these it can be seen that there is considerable variation between the various methods and even with the same method.

Table 1.

This Table gives the measurements of liver blood flow in the 'normal' dog, giving the names of the workers concerned, the technique and anaesthesia employed. The mean values vary considerably even with the same technique and anaesthesia but many of them give a value of the same order. In the individual groups of experiments the range of results is wide and indeed in some cases it would appear as if the result based on a value for liver blood flow as a percentage of the body weight would be as good an estimate. This does not invalidate the results given but only serves to emphasise how difficult is the problem of estimating liver blood flow, whatever technique is used.

Measurements of Hepatic Blood Flow in the 'Normal' Dog

Author & Date	No. of Animals	No. of Observations	Technique	Anaesthesia	Liver Blood Flow	
					Mean Ml/Kg/Min.	Range Ml/Kg/Min.
Burton-Opitz (1911)	15	?	Stromuhr	Ether	25	-
McLeod & Pearce (1914)	7	7	Collection	Ether	44	26 - 85
Grab, Janssen and Rein (1929)	5	5	Stromuhr	None	25	22 - 43
	6	6	Stromuhr	None	25	18 - 33
Herrick, Mann, Essex and Baldes (1934)	1	3	Stromuhr	None	62 ✻	52 - 71
	-	3	Stromuhr	None	103 ∓	84 - 114
Blalock & Mason (1936)	10	13	Collection	None	28.6	18.7 - 39
Soskin, Essex, Herrick and Mann (1938)	1	3	Stromuhr	Amytal	24.1	23 - 25
Grindlay, Herrick and Mann (1941)	8	66	Stromuhr	Barbiturates	24	16.8 - 30
	2	2	Stromuhr	None	22	-
Grodins, Osborne, Ivy and Goldman (1941)	5	14	Stromuhr	Barbiturates	15.5 ø	5 - 37
	21	46	Stromuhr	Barbiturates	6.6 u	2.3 - 13.8
Lipscomb & Crandall (1947)	20	20	BSP	None	32	18 - 48
Werner & Horvath (1952)	17	49	BSP	Barbiturates	42 ± 2.9	16 - 111

Author & Date	No. of Animals	No. of Observations	Technique	Anaesthesia	Liver Blood Flow	
					Mean Ml/Kg/Min.	Range Ml/Kg/Min.
Werner, MacCanon and Horvath (1952)	14	41	BSP	Barbiturates	40	-
Hallett, Holton, Paterson & Schilling (1952)	14	14	BSP	Barbiturates	56	31 - 93
Pratt, Burdick and Holmes (1952)	14	51	BSP	None	48.6 ± 9.9	37.7 - 65.4
Heinemann, Smythe and Marks (1953)	9	9	BSP	Barbiturates	27.6 ± 5	16 - 36
Smythe, Heinemann and Bradley (1953)	26	26	BSP	Barbiturates	29.5 ± 9.3	11.6 - 49.4
Smythe, Gilmore and Handford (1953)	27	27	BSP	Barbiturates	32 ±11.5	14 - 57
Selkurt (1953)	9	63	BSP	Barbiturates	35.4	21 - 55
Bollman, Khattab, Thors and Grindlay (1953)	?	35	BSP	None	42.5 ± 0.9	-

Author & Date	No. of Animals	No. of Observations	Technique	Anaesthesia	Liver Blood Flow	
					Mean Ml/Kg/Min.	Range Ml/Kg/Min.
Casselman and Rappaport (1954)	21	33	BSP	Barbiturates	37 ± 2.3	-
Selkurt (1954)	14	274	BSP	Barbiturates	35	-
	-	-	Direct	Barbiturates	33	-
Hamrick and Myers (1955)	13	13	BSP	Chloralose	47 ± 4.8	27 - 79
	5	5	BSP	Chloralose	52 ± 10.6	20 - 86
Smythe and Gilmore (1955)	14	14	BSP	Barbiturates	30 ± 11.5	16 - 60
Simpson and Saperstein (1955)	10	10	Rose Bengal	Barbiturates	31.5 ± 4.1	-
Fisher, Russ, Selker and Fedor (1955)	20	20	BSP	Barbiturates	49 ± 7.4	36 - 65
	10	10	BSP	Ether	70 ± 4.1	52 - 86
	20	20	BSP	None	45 ± 9.3	34 - 70

⋇ Fasted ± Fed BSP Bromsulphthalein
ø Portal u Hepatic Artery

X. METHODS

A. CHARACTERISTICSOF BROMSULPHTHALEIN

B. ESTIMATION OF BROMSULPHTHALEIN

C. SURGICAL PROCEDURES

The characteristics of the dye bromsulphthalein which has been used throughout this work and its estimation are dealt with in this section. The methods used for the determination of the content of dye present in the plasma, blood, liver, bile and urine given here have been used to obtain the values given in other sections. The usual method of obtaining the various samples or specimens in different animal species has been given here and any alteration of these practices is indicated in the relevant sections. The operative techniques used are given in this section but minor variations mentioned in subsequent sections. Several different types of animals have been used but an endeavour has been made to obtain as standard conditions as possible so that a comparison may be drawn from the results.

BROMSULPHTHALEIN SOLUTIONS

In the present study bromsulphthalein solutions have either been made up from bromsulphthalein powder with distilled or sterile water, or as supplied by Savory and Moore Ltd., in ampoules as a 5% solution (3 ml. ampoules containing 50 mgs. per 100 ml.).

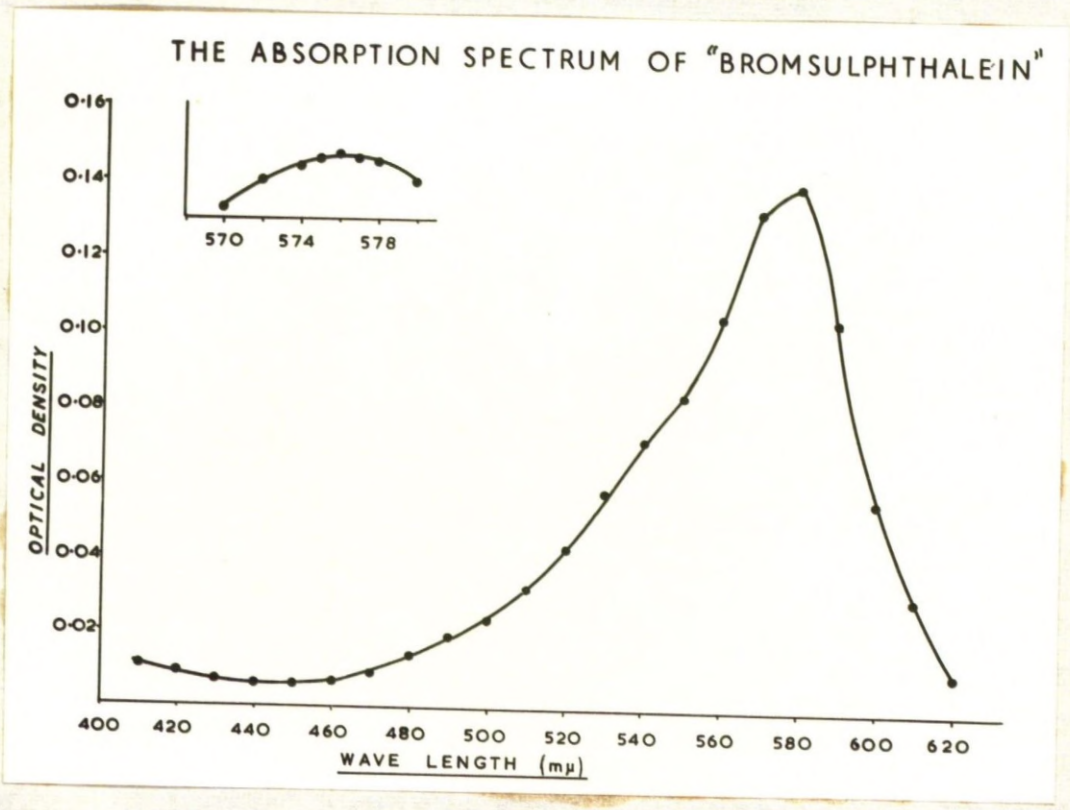
SOME BASIC PROPERTIES OF BROMSULPHTHALEIN

When alkali is added to bromsulphthalein (either powder or as an aqueous solution) a typical purple colour develops. The absorption spectrum of this solution is shown in Figure 18. The peak of the spectrum is reached between 570mμ(5700 A°) and 580mμ(5800 A°) the highest individual reading being obtained at 576mμ(5760 A°) (as shown in the insert Figure 18) when the pH of the solution is between 11 and 12. The purple colour which develops on the addition of weak solutions of alkali to an aqueous solution of bromsulphthalein is stable under normal laboratory conditions for at least an hour even with bright sunlight present. It has, however, been the practice to determine the content of dye in a solution as soon as possible after the addition of alkali (usually within five minutes) in order to avoid errors in determination.

Bromsulphthalein combines readily with protein and in the body this protein is usually an albumin (Brauer and Pessotti, 1948). The union between protein and dye is relatively loose since the addition of alkali to this complex results in the 'splitting off' of the bromsulphthalein to yield the typical

Figure 18.

The bromsulphthalein absorption spectrum.


THE ABSORPTION SPECTRUM OF "BROMSULPHTHALEIN"
OPTICAL DENSITY
0·16
0·14
0·12
0·10
0·08
0·06
0·04
0·02
570
574
578
400
420
440
460
480
500
520
540
560
580
600
620
WAVE LENGTH (mμ)

purple coloured solution. This solution, however, is much less stable than an aqueous solution of the dye. The peak of the absorption spectrum in time is gradually altered as the solution changes from purple to red, then yellow and finally becomes colourless.

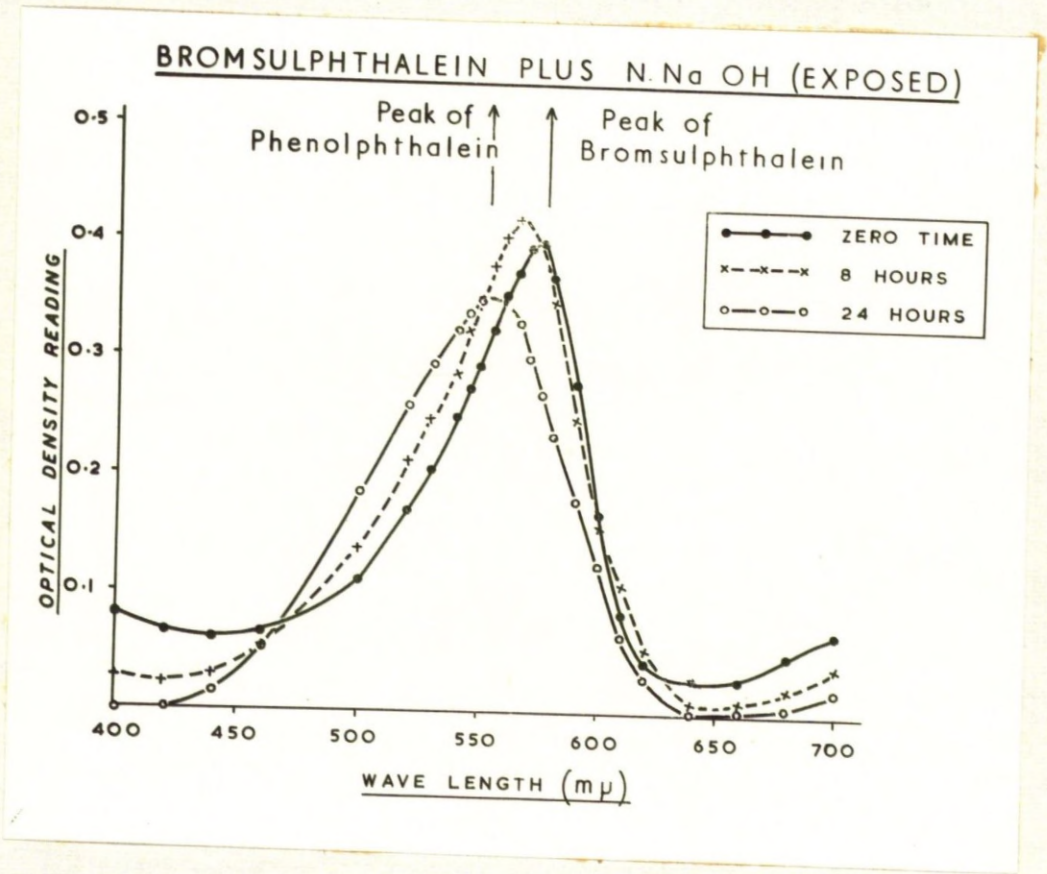
These changes take a considerable time with weak solutions of alkali, (several days) but with strong solutions of alkali (2 N and stronger) it occurs more rapidly (within 12 to 18 hours). Figure 19 shows the graphical results of the addition of N. NaOH to a bromsulphthalein solution. The peak of the spectrum approximates in time nearer and nearer to that of phenolphthalein (peak 552 mμ). The absorption spectrum initially characteristic for bromsulphthalein changes to that of a bromsulphthalein-phenolphthalein mixture which at first contains more bromsulphthalein than phenolphthalein and later the reverse. The proportions of each dye at any time will vary, depending upon the rate of breakdown of bromsulphthalein. The rate of the breakdown depends to some extent on the strength of alkali, the temperature and the time allowed for this process to occur.

Originally the alkali used for determining the quantity of dye present in a solution was 0.1N Sodium

Figure 19.

The effect of strong alkali (N. NaOH) on a bromsulphthalein solution. The solution is gradually broken down so that the solution is no longer a pure bromsulphthalein solution plus alkali but a combination of bromsulphthalein and its breakdown products. It can be seen that the absorption spectrum peak is altering with an increase in the strength of the alkali and on exposure to light and a rise of temperature.

If left exposed the solution eventually becomes colourless and there is no longer any reading within the range covered.


BROMSULPHTHALEIN PLUS N.Na OH (EXPOSED)
Peak of Phenolphthalein
Peak of Bromsulphthalein
ZERO TIME
8 HOURS
24 HOURS
OPTICAL DENSITY READING
0·5
0·4
0·3
0·2
0·1
400
450
500
550
600
650
700
WAVE LENGTH (mμ)

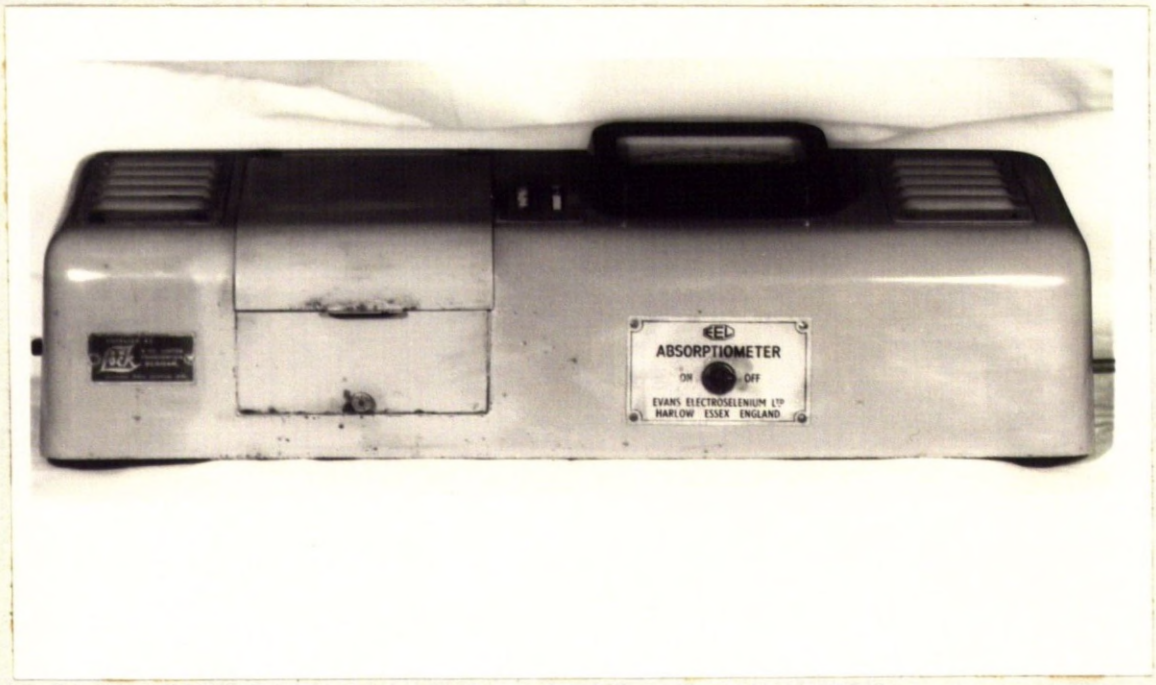
hydroxide, since this is used for the clinical test. It was found, however, that the addition of sodium hydroxide to various tissue-protein bromsulphthalein complexes resulted in the usual purple colour but it was often cloudy or opalescent. This is presumably due to the action of sodium hydroxide on any lipoid material present in the tissues used with the formation of "soaps" since it did not occur with individual proteins or pure protein mixtures. Since the presence of an opalescent or cloudy solution affects the colorimetric estimation of bromsulphthalein, sodium hydroxide was discarded as the routine alkali. Ammonium hydroxide has been found (by a process of trial and error) to be the most suitable alkali, since it did not produce any opalescent or cloudy solution when added to the tissue-protein bromsulphthalein complexes under examination in this work. Half-normal ammonium hydroxide has been used throughout since it has been found to be equivalent for stability and estimation purposes to 0.1N sodium hydroxide which is used clinically.

B. ESTIMATION OF BROMSULPHTHALEIN

Figure 20.

A photograph of the EEL Absorptiometer.

The various parts of this apparatus are described in the text.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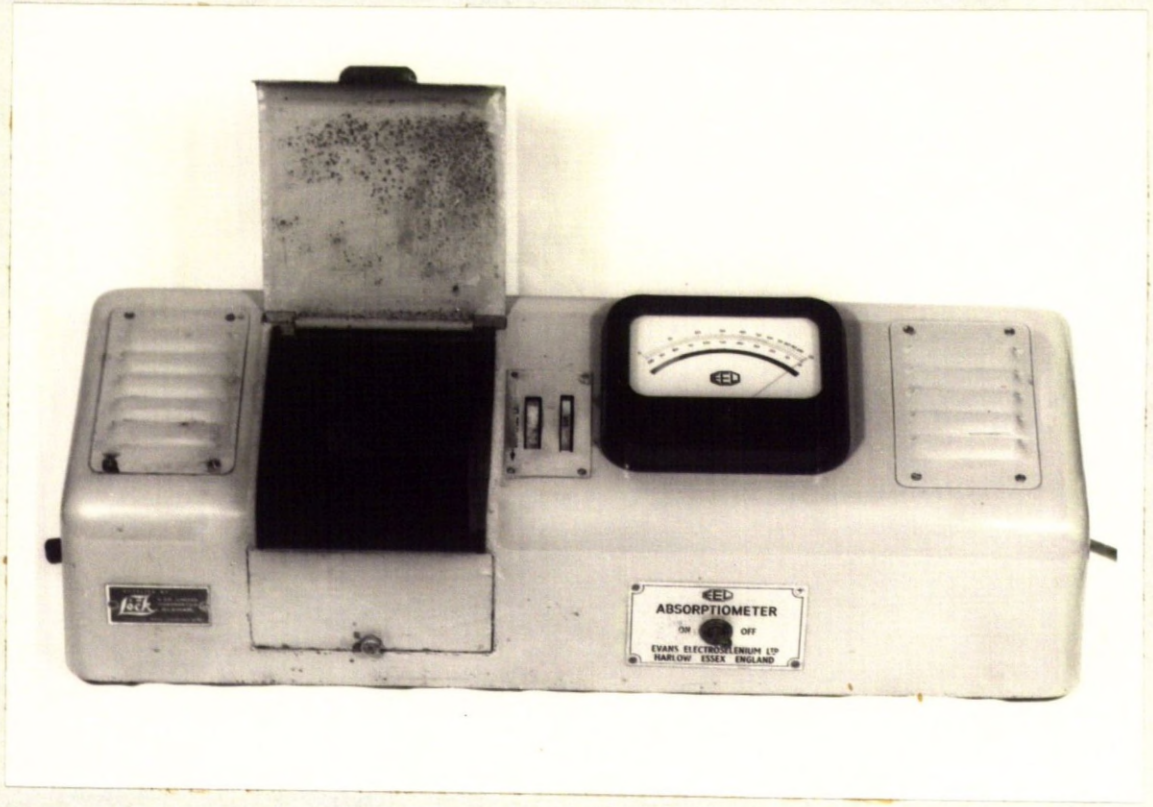
HARLOW ESSEX ENGLAND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EVANS ELECTROSELENIUM LTD
HARLOW ESSEX ENGLAND

THE ESTIMATION OF BROMSULPHTHALEIN

Bromsulphthalein in Aqueous Solutions.

The determination of the quantity of bromsulphthalein in a solution has been carried out by photoelectric methods. An EEL absorptiometer with fixed filters has been used in this study, but any similar machine may be used. The regression formulae may be derived in a similar manner although the constants will vary from machine to machine as do the wavelengths of the fixed filters. A photograph of the EEL absorptiometer is shown in Figure 20. The various parts of the apparatus can easily be seen. It has an enamelled cast aluminium case with a light-tight lid. It has a built-in stabilising mains transformer to allow for voltage fluctuations up to a plus or minus 10%. It also has external terminals for connections to a 12 volt battery, although these were never used in this study. The lamp is a 12 volt, 24 watt bulb. It has a condenser lens and heat absorbing filter. There are nine optical colour filters mounted in a wheel, the respective colours and wave-lengths of these filters are given below.

There is a zero adjustment wheel and it and the filter wheel protrude slightly above the case to give finger-tip control. The barrier-layer photoelectric cell is connected to a four-inch microammeter with an inverse logarithmic (density scale). The glass cells of fused glass contain 12.5 ml. of fluid and have an optical path of 20 mm. The cells containing test and control solutions can be moved into the light beam by a traverse mechanism operated from a lever at the side of the case. The numbers of the fixed filters with their corresponding colour and peak wavelengths for the EEL absorptiometer are as follows :-

Fixed filter number	Colour	Peak wave-length (A°)
609	Deep red	6900
608	Red	6600
607	Orange	6000
606	Yellow	5800
605	Yellow-green	5500
604	Green	5200
603	Blue-green	4900
602	Blue	4700
601	Violet	4250

The principle of the machine is quite simple. A beam of light is passed through the solution under examination and, after passing through a filter, concentrated on the photo-electric cell which gives a reading on the arbitrary scale. If the control solution is made to read zero on the scale then the reading of the test solution can be compared with this zero. Using a standard control solution and test solutions of known concentration it is possible to obtain calibration graphs and from these derive a conversion factor to convert any reading into a concentration.

For bromsulphthalein, solutions of known concentration were diluted and compared with a control solution of water using the 606 filter. This filter is chosen because it is nearest to the peak wavelength of bromsulphthalein.

It follows that :-

(the reading obtained using filter 606) x (the Dilution of the solution) x C.F. = concentration (mg./100 ml.) where C.F. is the conversion factor.

Therefore the conversion factor is equal to :-

$$\frac{\text{Concentration (mg./100 ml.)}}{\text{(Reading at 606) x(Dilution)}}$$

Since solutions of known strength and dilution are used all these individual items are known. By carrying out many of these estimations with different concentrations the conversion factor can be obtained with considerable accuracy (Figure 21). During the course of this study the value of this constant has been checked at regular intervals. The value of the conversion factor is 0.0086 to the nearest four figures.

Bromsulphthalein in the plasma and blood.

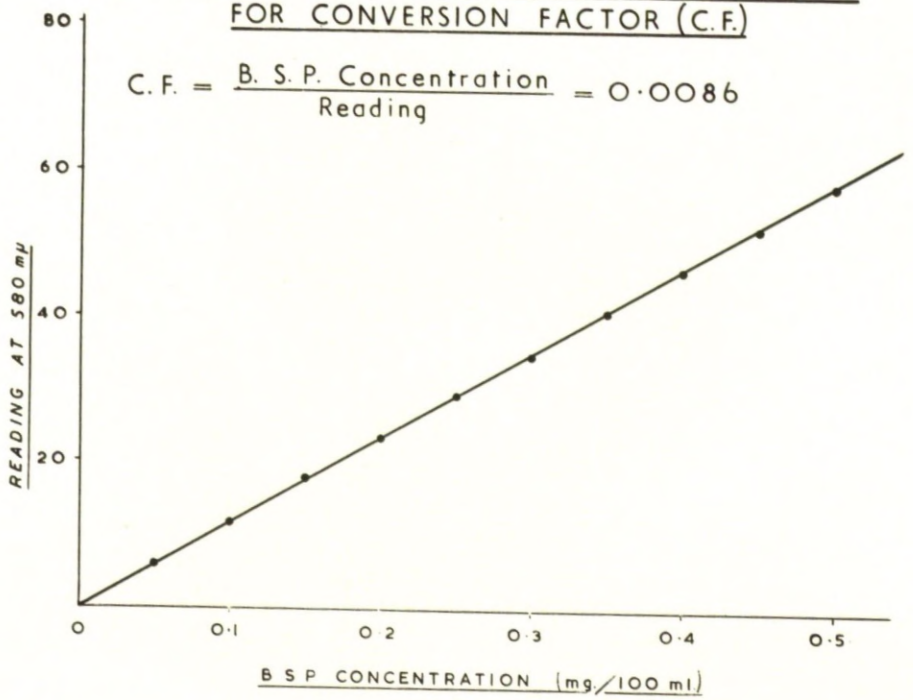
It is possible to determine the concentration of bromsulphthalein in a solution of plasma by simple comparison. A control specimen of plasma of the same species, preferably the same individual, is used. This can normally be obtained by taking a sample of plasma prior to the injection of bromsulphthalein. Provided the control solution is diluted similarly to the unknown solution then the only difference between the solutions is that one contains bromsulphthalein and the other does not. If the EEL absorptiometer is then "zeroed" on the control solution using the 606 filter then the reading for the unknown solution can be determined. If the reading of the unknown solution is multiplied by the dilution of the solution and the conversion factor it will give the concentration of

Figure 21.

The Calibration curve of bromsulphthalein at 5800 A^{O} (580 $m\mu$)

BROMSULPHTHALEIN (B. S. P.) CALIBRATION CURVE FOR CONVERSION FACTOR (C.F.)

$$C.F. = \frac{\text{B. S. P. Concentration}}{\text{Reading}} = 0{\cdot}0086$$



bromsulphthalein in the unknown solution in mg./100 ml. of plasma. The two samples are, however, only strictly comparable if there is no haemolysis present in either sample. If this method is used when serial samples are taken, then any sample which has any haemolysis present has to be discarded, and if the control sample has any haemolysis present then the whole set of samples are useless. This is obviously not very satisfactory for work involving serial plasma determinations, although it is still the usual method for the clinical test which involves taking a control sample and one or two unknown solutions at definite times following the injection of bromsulphthalein. Another method used to overcome this difficulty has been to compare the unknown solution with one containing a known quantity of bromsulphthalein.

In view of the difficulties mentioned in the previous paragraph, Gaebler (1945) described a method for the determination of bromsulphthalein in the presence of turbid, haemolysed or icteric serum. The principle of this method depends upon the use of a standard control such as distilled water and deriving a regression formula using two separate wavelengths close to each other. Using solutions of bromsulph-

thalein and haemolysed turbid of icteric serum of known concentrations separately and combined at these two wavelengths it is possible to obtain the necessary constant for the machine and filters used in order to eliminate the effects of haemolysis. A similar method has been employed here except that the filters have been chosen so that one corresponds closely to the peak of bromsulphthalein and the other to the peak of alkaline haematin solution. On the EEL absorptiometer this means using the filters 606 (5800A^{o}) and 601 (4250A^{o}) respectively. The readings using the filter 606 will thus be predominantly due to haemoglobin when a haemolysed specimen of plasma containing bromsulphthalein is examined.

The derivation of the regression formula for the determination of bromsulphthalein in the plasma in the presence of haemolysis.

The observed reading at 606 and 601 filters respectively in the haemolysed plasma solution are called R 606 and R 601 respectively. The true readings for bromsulphthalein (i.e. the reading that one would have obtained from an aqueous solution) using these filters are called 606b and 601b respectively. The corresponding readings for the

haemolysed plasma alone (i.e. without any bromsulphthalein) are called 606h and 601h. It is first of all necessary to determine experimentally the ratio of 606b and 601b and also the ratio of 606h and 601h. Figure 22 and Figure 23 show a typical representative of the graphs from which these respective ratios have been derived. Figure 22 shows the readings obtained using the 606 and 601 filters of several known concentrations of bromsulphthalein. Figure 23 shows similar readings for alkaline haemoglobin solutions but all containing less than 0.3 gm./100 ml. of haemoglobin. The amount of haemoglobin in the original solution was always determined separately. These estimations were carried out many times during this work, especially after every servicing of the absorptiometer and although slight variation has been found it was not sufficient to have affected the ratio obtained initially to any appreciable extent.

In Figure 24, it can be seen that all the readings of alkaline haemoglobin solutions of known strength fall on a straight line initially but later the readings of the haemoglobin solutions above the concentration of 0.3 Gm./100 ml. of haemoglobin fail to fall on a straight line. With the standard

Figure 22.

Calibration curve of bromsulphthalein at 425mμand 580mμusing the EEL absorptiometer (with fixed filters 601 and 606 respectively).

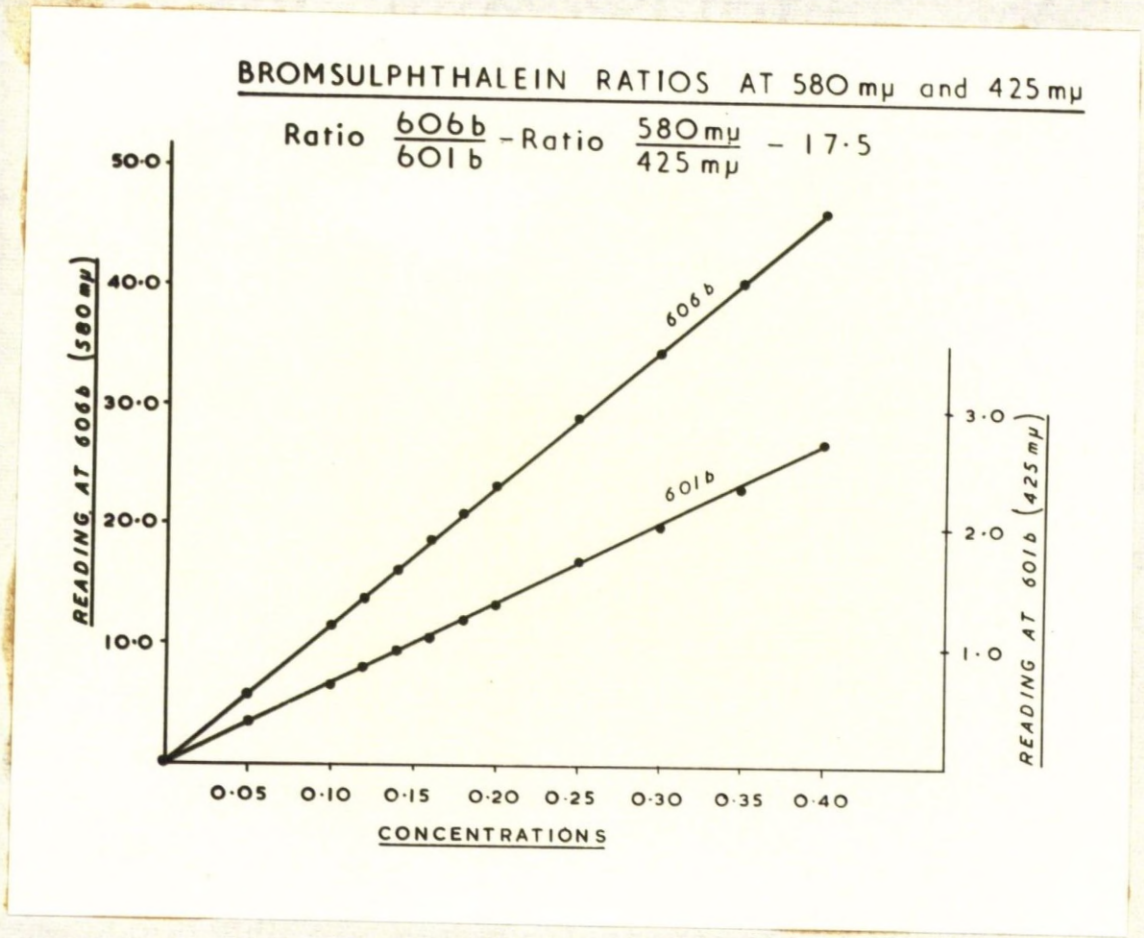

BROMSULPHTHALEIN RATIOS AT 580 mμ and 425 mμ
Ratio 606b/601b - Ratio 580mμ/425 mμ - 17·5
50·0
40·0
30·0
20·0
10·0
READING AT 606b (580 mμ)
606b
601b
3·0
2·0
1·0
READING AT 601b (425 mμ)
0·05
0·10
0·15
0·20
0·25
0·30
0·35
0·40
CONCENTRATIONS

Figure 23.

Calibration curve of alkaline (NH_4OH) haemoglobin using the fixed filters 601 (425mμ) and 606 (580mμ).

ALKALINE HAEMOGLOBIN SOLUTION
CALIBRATION AT 425mμ and 580mμ

$$\frac{601h}{606h} = \frac{425m\mu}{580m\mu} = 3 \cdot 6$$

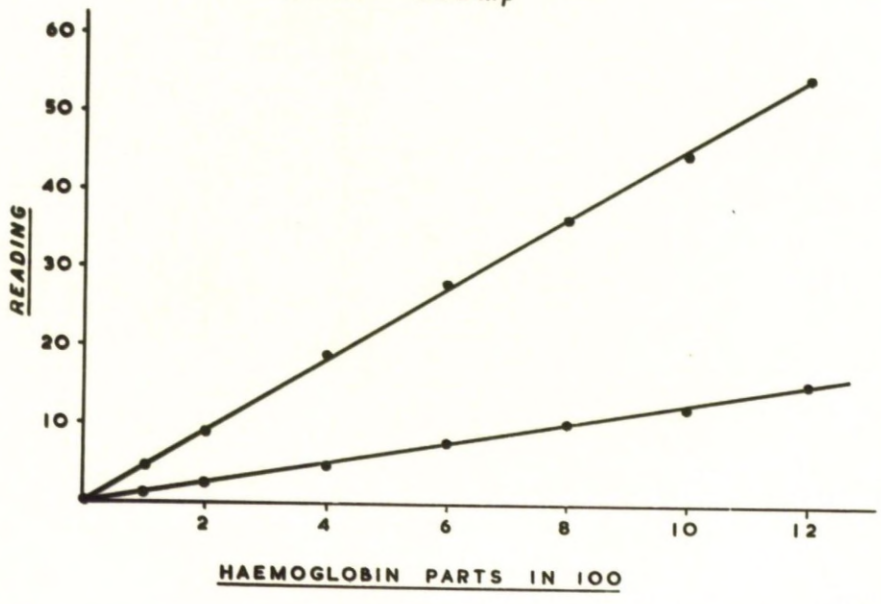
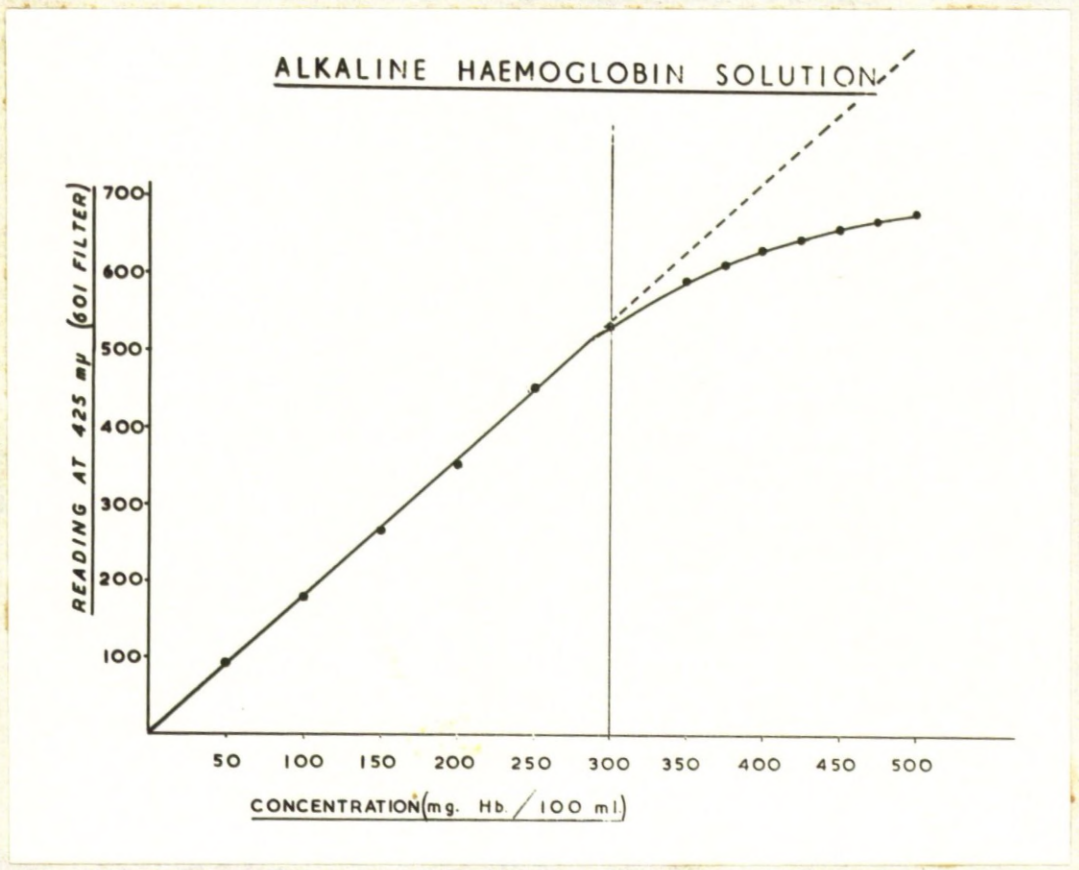


Figure 24.

The optical density readings of known concentrations of alkaline haemoglobin solutions using the 601 filter of the EEL absorptiometer.

It can be seen that in the initial phase the readings approximate to a straight line, although later they fail to do so and fall on a curved line.


ALKALINE HAEMOGLOBIN SOLUTION
READING AT 425 mμ (601 FILTER)
700
600
500
400
300
200
100
50
100
150
200
250
300
350
400
450
500
CONCENTRATION (mg. Hb. / 100 ml.)

dilutions used in the EEL absorptiometer for plasma samples this concentration of haemoglobin gives a reading between 30 and 35 using the 601 filter and therefore any solution examined which gives a reading above this required further dilution was considered unsuitable for determination. Very few specimens with this degree of haemolysis were encountered in the course of this work and, if of this degree, they were usually discarded.

The subsequent arithmetic for the derivation of a regression formula depends entirely on these ratios (and hence the importance of performing a large number of determinations) since the resulting equation can be no more accurate than these preliminary results allow. Several separate determinations of these ratios have been carried out because of this fact and especially on different samples of plasma from various animal species. As a result of this initial study the regression formula was found to be suitable for the animal species studied here, with the exception of the human subject.

It turns out that these ratios determined experimentally are :-

606b = 17.5 x 601b or 601b = 0.06 606b

and 601h = 3.6 x 606h or 606h = 0.28 601h

The observed reading of bromsulphthalein (BSP) in a haemolysed solution is the sum of that due to bromsulphthalein alone and alkaline haematin alone whether the reading is taken using filters 601 or 606.

It follows therefore that :-

R 606 = 606b + 606h

= 606b + 0.28 601h

and 606b = R606 — 0.28 601h .....(I)

Also R601 = 601h + 601 b

= 601h + 0.06 606b

or 0.06 606b = R601 — 601h .....(2)

Multiplying (2) by 0.28 we get :-

(0.06) (0.28) 606b = 0.28 R 601 — 0.28 601h..(3)

Subtracting (3) from (I) to eliminate 601h we get :-

606b (1 — 0.02) = R 606 — 0.28 R 601

i.e. 0.98 606b = R 606 — 0.28 R 601

Dividing by 0.98 we find that :-

606b = 1.02 R606 — 0.29 R601 .....(4)

Equation (4) therefore gives the "true" aqueous value for bromsulphthalein in terms of the "mixed" readings using filters 606 and 601.

Expressed with reference to the wavelengths, the formula is

1.02 x reading at 5800A° - 0.29 x reading at 4250A°
= True reading of bromsulphthalein at 5800A°

Once the "true" aqueous value has been obtained from this regression formula it can be multiplied by the dilution of the solution and the conversion factor (C.F.) to give the concentration of bromsulphthalein in the plasma sample being examined. This can be put down thus :-

"True" aqueous reading for bromsulphthalein x Dilution x C.F. = Concentration (mg/100 ml.)

This regression formula has been used as a routine for the determination of the concentration of bromsulphthalein in plasma.

The estimation of the plasma concentration of bromsulphthalein in practice.

All blood samples were centrifuged and 1.0 ml. of the supernatant plasma was removed and diluted 15 times with 0.5 N NH_4 OH and read against distilled

water in the EEL single celled absorptiometer at wavelengths of 5800A° (606 filter) and 4250A° (601 filter). The readings at these wavelengths are inserted into the regression formula derived above, viz.

1.02 x Reading at 5800A° - 0.29 x Reading at 4250A° = True reading of bromsulphthalein at 5800A°

In this way, errors due to haemolysis (up to 0.3 gm./100 ml. plasma) are avoided. The bromsulphthalein reading without errors due to haemolysis can now be multiplied by the conversion factor (obtained originally from the calibration curve as explained above) to express the reading as mgm. BSP /100 ml. plasma.

The estimation of the content of bromsulphthalein in the blood.

In order to obtain the actual amount of dye present in the plasma it is necessary to determine the plasma volume or to take it as a fixed proportion of the body weight. Since several animal species have been used in the work it has been the standard practice to base the plasma volume upon the animals weight. It is realised that this will tend to overestimate the plasma volume in the heavier animals of a particular species and underestimate it in the small ones, but at

least it should be comparable in different species. The standard value for plasma volume has been taken as 50 ml. per Kg. body. Using this standard figure the content of dye can be calculated once the concentration of dye is determined.

Since bromsulphthalein normally combines with the albumin fraction of the plasma (Brauer and Pessotti, 1948, 1949; Ingelfinger, Bradley, Mendeloff and Kramer, 1948) the content of dye present in the plasma is equal to the content of dye in the blood.

Bromsulphthalein in the bile

The bile obtained in special graduated test tubes during the course of some of these experiments, was usually collected over time intervals of ten or fifteen minutes. To each specimen 10 ml. of distilled water was added and duplicate samples (usually 0.5 or 1.0 ml.) were taken. One of these samples was further diluted with N/2 NH_4OH, and to the other was added an equal quantity of distilled water (to which one drop of N.HCl had been added) The two samples were compared directly in the absorptiometer and again the concentration of bromsulphthalein in mg./100 ml. given using the conversion factor obtained from the calibration curve. Since the volume of bile specimen

plus 10 ml. of distilled water is given in the calibrated test tubes for collecting the bile specimens, the actual content of dye present can be readily calculated.

Bromsulphthalein in the urine.

The urine samples were centrifuged, and the concentration of bromsulphthalein in the supernatant fluid determined in a similar manner to that described above for bile. No dilution of the supernatant urine was required since the amount of dye present was found to be small under the experimental conditions obtaining in this work.

Bromsulphthalein in the liver

In some experiments the content of dye present in the liver was determined. The animal was killed and the liver removed immediately. The estimation of the terminal content of dye in the liver consisted essentially of three stages :-

(i) The liver was weighed and several 30 mg. samples were ground up separately with dry sand. To the macerated specimen equal quantities (125 ml.) of absolute alcohol and ether were added with 3 ml. of N.HCl, and the mixture shaken. The supernatant fluid containing lipids, pigments and blood were

filtered off, and a further quantity of the acidified alcohol-ether mixture added, followed by filtration. This procedure was repeated until the solid material was cleared of pigments.

(ii) To the cleared liver substrate 200-300 mls. N/2 NH_4OH was added, the mixture shaken and allowed to stand. The fluid was decanted and a further volume of alkali until no further colour was extracted.

(iii) The cloudy purple alkaline fluid was then filtered through on a Whatman's No. 1 filter to yield a clear purple solution. The concentration of bromsulphthalein in specimens of this solution was measured against distilled water. The readings were taken at the same wavelengths as for plasma and a similar regression formula used, (see later). The concentration of bromsulphthalein in the clear solution is given from the calibration curve and thus the content of bromsulphthalein obtained. The average bromsulphthalein content of the several samples is used to obtain the total liver content.

The <u>liver regression formula</u> is derived in a similar manner to that for the plasma regression formula described previously. Instead of using an alkalinised haemoglobin, an alkalinised liver solution is used.

The liver solution is obtained by using normal liver and subjecting it to the processes described above. The ratio of the readings obtained experimentally using the 601 and 606 filters for this solution are determined at various dilutions, Figure 25. Using specimens from many different livers the ratio of 601 to 606 was 2.86 to 1. The regression formula thus works out as follows :-

1.02 x reading using the 601 filter (5800A°)
- 0.34 x reading using the 606 filter (4250A°)
= True reading of bromsulphthalein at 5800A°.

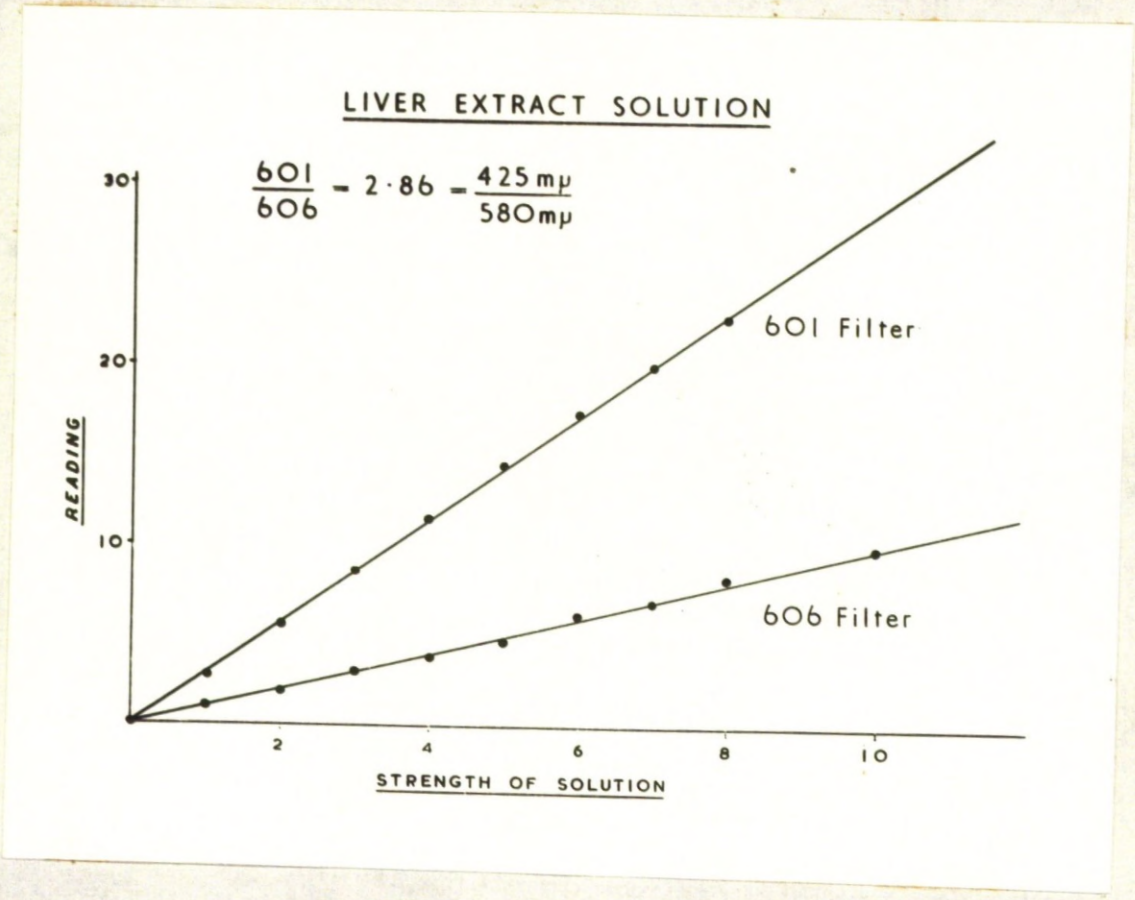
The accuracy of the formula depends upon the same factors described for the plasma regression formula and was checked at regular intervals.

The determination of the presence of bromsulphthalein in the liver is then carried out as follows :-

The above method is used to obtain the bromsulphthalein containing extract. This solution, after suitable dilution, is compared with distilled water as a control in the EEL absorptiometer using the 601 and 606 filters. The readings obtained are then used in the regression formula derived as mentioned above. Once the true reading at 5800A° (filter 606) has been obtained it is multiplied by

Figure 25.

The ratio of 'liver solutions' treated by the Extraction process using the 601 and 606 filters of the EEL absorptiometer.


LIVER EXTRACT SOLUTION
601/606 = 2·86 = 425mμ/580mμ
30
20
10
READING
601 Filter
606 Filter
2
4
6
8
10
STRENGTH OF SOLUTION

the dilution and the conversion factor (0.0086) to give the concentration of bromsulphthalein (mg./100 ml.) present in the fluid extracted from the liver. Since the volume of this extract and the amount of liver tissue from which it had been obtained is known, the amount of bromsulphthalein per gram of liver can be obtained. Multiplying this by the weight of the liver in grams the actual quantity of dye in the liver can be determined. Since several small (20 - 30 gm.) portions from each liver are examined for bromsulphthalein, the result obtained is a good average of the quantity of dye present.

The actual amount of dye obtained is, however, only a fraction of that actually present, presumably due to the extraction process, as explained in the results. It varies a little with the individual species of animal e.g. in the cat it is approximately 50%, whereas in the dog it is approximately 30 - 40%. A range of the expected dye content of the liver can therefore only be given.

C. SURGICAL PROCEDURES.

ANAESTHESIA

The majority of experiments in cats, dogs and rats were carried out under anaesthesia. The experiments on goats, sheep and human subjects were carried out without anaesthesia and local anaesthesia was used for experiments on hens.

The standard anaesthetic used has been sodium pentobarbitone (Nembutal, May and Baker, Ltd.) given intravenously in a dose of 25 mg./Kg. body weight. In dogs, diethylthiambutene hydrochloride (Themalon, Burroughs Wellcome & Co.) 15 mg./Kg. body weight was usually given intramuscularly as pre-medication, thirty minutes prior to the Nembutal. Induction of anaesthesia in cats and rats was carried out with inhalation of ether and anaesthesia maintained with intravenous Nembutal. The local anaesthetic used for the experiments on hens was 1% lignocaine hydrochloride.

If the experiment was an acute one and the animal sacrificed afterwards, it was killed either by giving an overdose of Nembutal (intravenously) or an artificial pneumothorax was performed. When repeated experiments were made on a particular animal an antibiotic cover of penicillin with or without streptomycin

was given after each experiment and sufficient time allowed for complete recovery between experiments. Unless otherwise stated, all experiments were carried out on the fasting animal, i.e. no food for at least 12 hours.

BLOOD SAMPLES

All blood samples were collected in either paraffin-lined (all-glass) suringes or the syringes used had been washed out with a heparinised saline solution. The blood samples were immediately put into test tubes (suitable for insertion into a centrifuge) which contained 2 or 3 drops of heparin (5,000 units per ml.) and immediately shaken. The blood samples were then centrifuged in order to obtain the plasma for analysis. It was also possible to calculate the haematocrit (the ratio of cells to plasma) directly from these tubes after centrifuging. In cats, dogs and rats, the blood samples were usually obtained from a femoral vein or anterior jugular vein, mostly the former. A small incision was made directly over the vein under aseptic conditions and it was exposed by blunt dissection. The samples were then taken at the required times under direct vision with a 5 ml. syringe and a No. 1 gauge needle. After the needle

had been inserted into the lumen of the vein a blood sample was drawn into the syringe for an equal time (in seconds) before and after the specified minute. Pressure was applied to the vein for a short time after the removal of the needle to prevent haemorrhage. All times were taken on a stop-watch, started at the beginning of each experiment.

In goats and sheep, the blood samples were obtained from the anterior jugular vein. A large bore needle being inserted into the vein and a polythene (or nylon) tube of a smaller bore passed through the needle into the vein and the needle then withdrawn. The polythene tube was left in the vein until the end of the experiment and then removed. The tube was also washed out occasionally with a small quantity of heparinised saline in order to prevent clotting taking place in the tube between samples. A needle was inserted into the exposed end of the polythene and blood withdrawn as required, between samples a needle with a blocked end was inserted in order to avoid loss of blood.

A similar technique was used in hens, except that the polythene tube was inserted directly into a small vein in the wing. The skin over the vein was

anaesthetised with local anaesthetic (1% xylocaine) and the vein exposed by blunt dissection after incising over it.

In human subjects samples were taken by repeated venepuncture from a suitable vein in either forearm.

Samples of blood in certain experiments (on dogs) were collected from the hepatic veins and the inferior vena cava. In order to obtain these samples a cardiac catheter was inserted under aseptic conditions into either the brachial or anterior jugular vein. Under X-ray fluoroscopy the catheter was passed into the superior vena cava, the right auricle of the heart and out through the inferior vena cava into one of the hepatic veins. Blood samples were taken in a similar manner to that described above from the polythene tube used in goats and sheep.

OPERATIONS

The usual operation performed in cats, dogs and rats was to make an acute biliary fistula. Through a midline, right paramedian or right subcostal incision, the gall bladder and extra-hepatic bile ducts were exposed. The gall bladder (if present) was either re-

moved or the cystic duct ligated in two places after it had been freed from the liver. The common bile duct was then exposed and cleared from the surrounding connective tissue. A small incision made in the wall and a polythene tube inserted into the lumen and then securely ligated in position. The tube could then be brought out through the abdominal incision so that bile could be collected at regular intervals (usually every 10 or 15 minutes) in graduated test tubes. The abdominal wound was then sutured in layers and the animal allowed a period of time to recover from the immediate effects of the operation. At the end of each experiment the bile in the gall bladder was examined in order to make sure that no dye had leaked into the gall bladder.

In some animals, the ureters were also catheterised in order to obtain the urine secreted throughout the duration of the experiment. Often, however, all the urine present in the bladder was obtained at the end of an experiment by direct puncture (with a needle and syringe) of the bladder wall. In female animals, sometimes the method of choice was catheterisation of the bladder with a rubber or polythene tube at the beginning of the experiment and urine

collected throughout, or catheterisation carried out at the end of the experiment to obtain all the urine secreted.

In some experiments, the effect of an operation on the excretion of bromsulphthalein was determined. Either a laparotomy was performed, followed by immediate suturing, or a non-abdominal operation was carried out.

The animals were on an operating table fitted with a heating element so that a constant body temperature was attained throughout the experiment. A rectal thermometer was inserted at the beginning of each experiment and left in position in order to check that a constant temperature was maintained.

CONTINUOUS INFUSION EXPERIMENT

In the majority of experiments described in this work a single injection of bromsulphthalein has been given but in a few experiments a continuous infusion of bromsulphthalein was given. The continuous infusion machine used was a high-torque, constant speed electric motor, geared down to drive a micrometer syringe containing a solution of bromsulphthalein (50 mg./ml). Usually, a priming dose of the dye was given intravenously and a continuous intravenous infusion of

bromsulphthalein (0.035 - 0.120 mg./Kg./min.) was started. Blood samples were taken at various times until plasma bromsulphthalein equilibrium was established (a period which varied from one to four hours).

MATHEMATICAL TREATMENT OF THE RESULTS

A mathematical treatment of the results is given in the Results and again in an Appendix. The application of this in a simplified form is given in the results, where it can be more easily dealt with than at present.

EFFECTS OF VARIOUS SUBSTANCES ON THE BROMSULPHTHALEIN DISAPPEARANCE CURVE

The routes of administration of these substances has been either by mouth, stomach tube, or by intravenous injection. The doses and nature of these substances as well as the time of administration are given fully in the relevant sections of the results.

XI. RESULTS

A. A study of the temporal distribution of bromsulphthalein and the derivation of a modified bromsulphthalein liver test.

PRELIMINARY EXPERIMENTS

1. Experiments on Rats

Prior to determining the distribution of bromsulphthalein in the dog, preliminary experiments were carried out on rats and cats.

The rat experiments were mainly concerned with the collection of bile. Rats were anaesthetised with ether and maintained in the anaesthetised state with intravenous 'Nembutal' and/or 'Themalon'. An acute biliary fistula was performed and the main bile duct cannulated, the subsidiary branches being ligated. In these animals, there is no gall bladder. A quantity of bromsulphthalein, either 2 or 5 mgs./Kg. body weight, was given intravenously into a femoral vein which had previously been exposed. The amount of dye recovered over different intervals of time was then determined. In the rats, it was found that bromsulphthalein appeared rapidly in the bile so that after 30 minutes, at least 50% of the dose given could be recovered and 80% or over of the dye was recovered in 45 - 60 minutes. On no occasion, however, was there a 100% recovery, even when the experiments lasted for several hours.

It was, however, felt for three reasons that these animals were unsuitable for determining temporal distribution of bromsulphthalein:-

(i) The small plasma volume of these animals meant that minute amounts of blood would be required for serial estimations of the concentration of bromsulphthalein present in the plasma, otherwise the animal wound be rendered hypovolaemic with all its inevitable circulatory changes. The estimation of bromsulphthalein in small quantities of blood containing a very low or minute concentration of dye would give unreliable results using colorimetric methods.

(ii) The obvious rapidity of the rate of removal of bromsulphthalein from the circulation by the liver, means that the liver initially contains a large percentage of the dose of bromsulphthalein given, only to rapidly excrete it into the bile. The estimate of the terminal quantity of dye becomes difficult because of the small amount present within the liver substance.

(iii) Since the solution of bromsulphthalein injected contains 50 mg./ml. any small error in giving exactly 2 or 5 mg./Kg. body weight

will be magnified even using a tuberculin type of syringe.

Because of these anticipated difficulties it was decided to conduct a series of experiments on a larger animal, such as the cat.

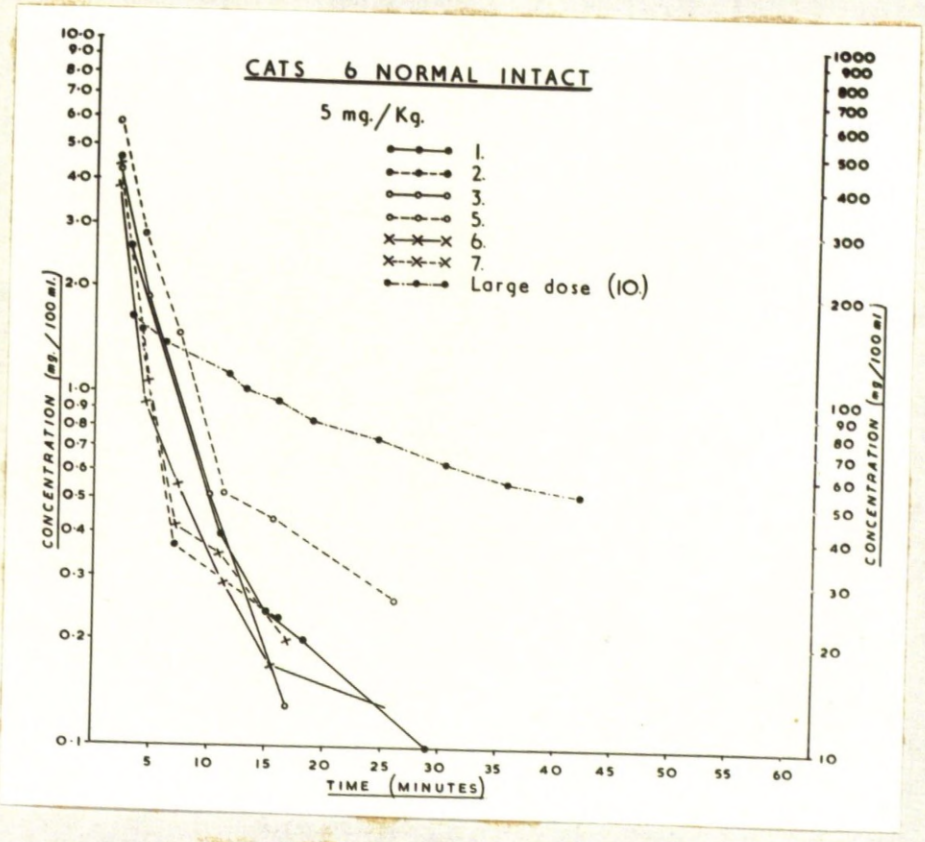
2. Experiments on Cats

The disappearance of bromsulphthalein from the blood was observed in 10 animals. In 7 of these cats, the disappearance of the dye from the blood following a 5 mg./Kg. dose was observed in the 'normal' cat, i.e. in the anaesthetised state, with a small superficial skin incision in each groin in order to expose the femoral veins. 2 cats had acute biliary fistulae performed as well as the femoral vein being exposed, the remaining cat was given a large dose of bromsulphthalein (200 mg., i.e. 63 mg./Kg. body weight). The content of bromsulphthalein in the liver was determined in 5 other cats, who were anaesthetised, given a dose of 20 milligrams of bromsulphthalein and killed 10 minutes later. The liver and bile ducts were then immediately removed and a sample of blood taken from the vena cava or heart.

The logarithm plasma concentrations of brom-

Figure 26

The logarithm plasma concentration of bromsulphthalein plotted against time (minutes) in 6 'Normal Intact' cats who had been given a 5mg./Kg. body weight dose of bromsulphthalein intravenously. Also shown are the concentrations obtained in Cat 10 who was given a large dose 200 mg. of bromsulphthalein, i.e. 63 mg./Kg. body weight. The concentrations are very high in this cat as indicated by the scale on the right-hand side of the graph.


CATS 6 NORMAL INTACT
5 mg./Kg.
1.
2.
3.
5.
6.
7.
Large dose (10.)
CONCENTRATION (mg./100 ml.)
TIME (MINUTES)

sulphthalein plotted against time are shown in 6 'normal' cats in Figure 26. It can be seen that the concentration falls rapidly from initial level at 2 - 3 minutes of 4.0 - 6.0 mg./100 ml. to 0.3 mg. /100 ml. and less after 12 - 15 minutes. The plotted concentrations appear to fall on a straight line initially with the suggestion of a change at low concentrations in some cases. Cat 4 could not be included in this series because it had just eaten, and the plasma specimens were opalescent, making it impossible to estimate the amount of dye present by any colorimetric technique. The other cats had been starved as were all the animals in subsequent series of experiments.

Also shown in Figure 26, are the plasma concentrations obtained in cat 10 who was given a large dose of bromsulphthalein. The value of the concentration for this particular cat is given on the right hand scale and it can be seen that initially at three and a quarter minutes the concentration was 164 mg./100 mls. The subsequent concentrations appeared to fall on a straight line, and at the end of the experiment at 42 minutes, the plasma concentration was still high, it being 55.6 mg./100 mls.

In this particular experiment, the blood concentration has fallen to approximately one-third of the initial concentration in a period of approximately 39 minutes. The subsequent plasma concentrations were not determined in this cat as it was killed in order to obtain the liver to test the method used for the recovery of bromsulphthalein in the liver, even though the exact quantity of dye present was not known.

In the case of cats 8 and 9, acute biliary fistula operations had been performed and the graphical results in these cases are given in Figures 27 and 28. Figure 28 shows the blood and bile content of dye over a period of 60 minutes. The 5 mg./Kg. dose given in this cat amounted to 15.5 mg. The plasma concentrations which were obtained at various times during the first 60 minute period of the experiment have been converted to the content present in the blood, based on the assumption that the plasma volume was 50 mls./Kg. body weight. It can be seen in Figure 27 that there is a rapid fall in the amount of dye present in the blood so that after 10 minutes, there is, in fact, a very small quantity of dye present, usually 0,1 mg. or less. At 60 minutes, the dye content of the plasma was only 0.02 mg. The amount

Figure 27

The content of bromsulphthalein in the blood and bile of cat 8. This cat had an acute biliary fistula operation and after a control period was given 5 mg./Kg. body weight of bromsulphthalein intravenously (i.e. 15.5 mg.) The blood content has been based on the observed plasma concentration and accepting the plasma volume as a fixed proportion of the body weight (50 ml./Kg. body weight). It is also assumed that at zero time the total dose of bromsulphthalein is present in the blood and at zero time there is no dye present in the bile.

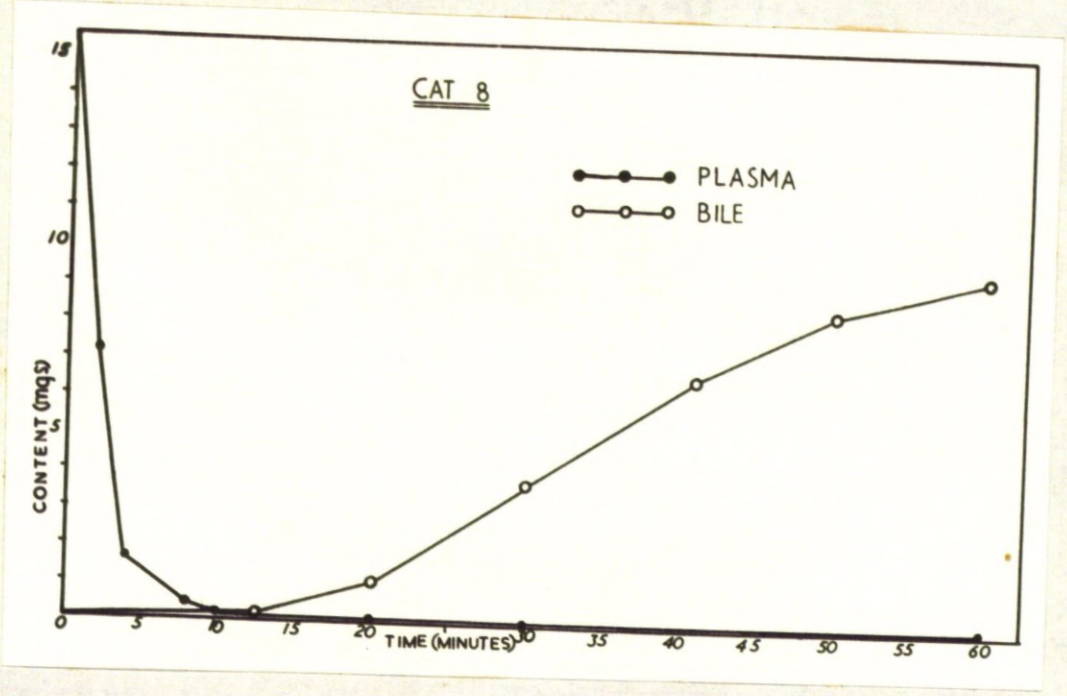
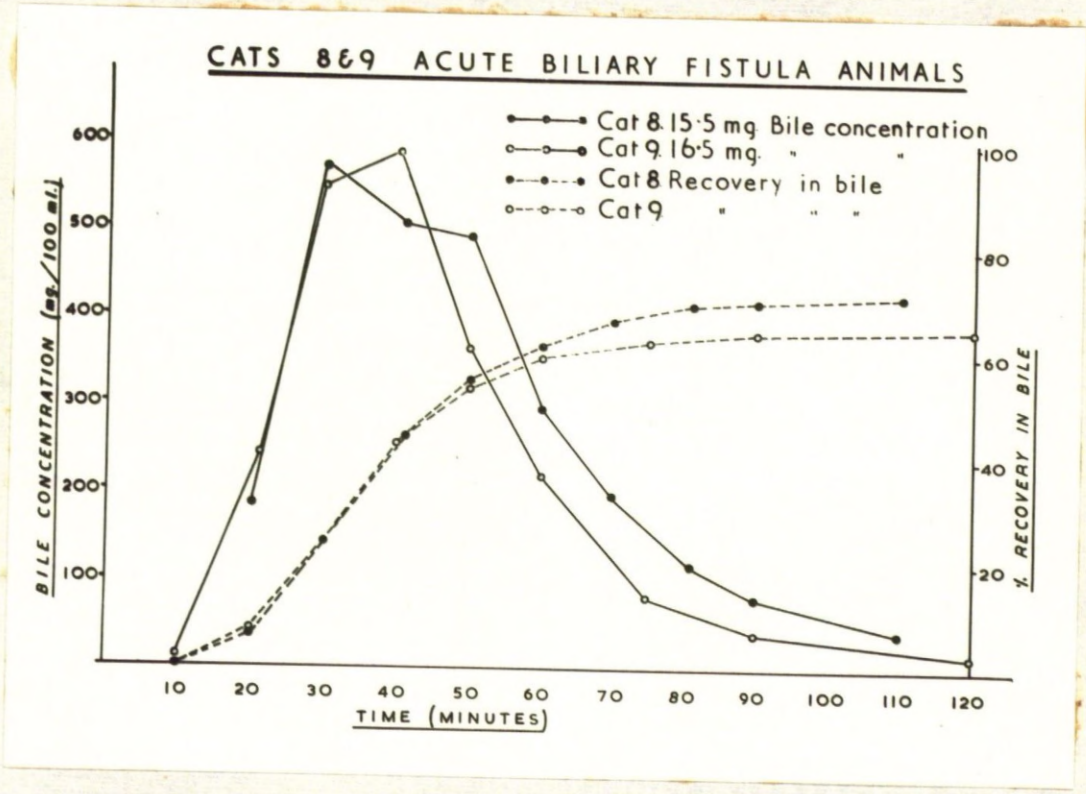

CAT 8
PLASMA
BILE
CONTENT (mgs)
15
10
5
0
5
10
15
20
TIME (MINUTES)
30
35
40
45
50
55
60

Figure 28

The concentration of bromsulphthalein in the bile in the acute biliary fistula Cats 8 and 9.
The percentage of the dose of bromsulphthalein given which has been recovered in the bile in relation to time (minutes) is given. After one hour in each cat approximately 60% of the dye has been recovered in the bile.


CATS 8&9 ACUTE BILIARY FISTULA ANIMALS
Cat 8. 15·5 mg. Bile concentration
Cat 9. 16·5 mg. " "
Cat 8 Recovery in bile
Cat 9 " "
BILE CONCENTRATION (mg./100 ml.)
% RECOVERY IN BILE
TIME (MINUTES)
600
500
400
300
200
100
100
80
60
40
20
10
20
30
40
50
60
70
80
90
100
110
120

of bromsulphthalein recovered in the bile is also given and it can be seen that as time proceeds, the amount of dye recovered increases progressively. There is a delay in the appearance of bromsulphthalein in the bile due to the inevitable dead space present in the biliary tree within, and outside, the liver substance, as well as the dead space in the cannular introduced into the common bile duct. In this particular experiment, even after twenty minutes, only 1 mg. of bromsulphthalein (about 6.5% of the dose given) had been recovered. At the end of 60 minutes, 9.5 mg. of bromsulphthalein had been recovered, that is 61.3% of the dose given. If one considers in the normal cat that bromsulphthalein is present within the blood, bile or liver, then from this graph it would appear that at 10 minutes the majority of the dye must be present within the liver. The actual quantity of dye in the blood and bile at this time was just under 0.14 mg. This is 0.9% of the quantity of dye originally injected. In cat 8, therefore, only 1% of the dye is in these two compartments and 99% of the dye given could be assumed to be in the liver, if these are the only three compartments of the body in which bromsulphthalein is distributed. Figure 28

shows the recovery of bromsulphthalein in the bile in both cat 8 and cat 9. It can be seen that the recovery of dye in cat 9 in the bile was slightly less than 60% at 60 minutes, but in [redacted] these experiments which were carried out for over a hundred minutes, the percentage recovery of dye in the bile in cat 8 was 71.4% at 110 minutes, and 65% in 120 minutes in the case of cat 9. Once again, however, it can be seen that there is a very small percentage of dye in the bile at 10 minutes and on this graph it is virtually a zero quantity and, in fact, there is still less than 10% of dye present at 20 minutes. It can be seen also, in this figure, that the bile concentration of bromsulphthalein in mg./100 mls. rises sharply to a maximum between 30 - 40 minutes, after which it falls to low values, although even at the end of 100 minutes, the concentration is of the order of 30 - 50 mg. of bromsulphthalein per 100 mls. of bile. Presumably, the maximum concentration of bromsulphthalein excretion from the liver cells into the bile biliary tree would occur a little time before 30 - 40 minutes, due to the biliary tree and cannula dead space of a collecting apparatus. There appears to be however, a good degree of uniformity in the behaviour

pattern of excretion of bromsulphthalein into the bile in these two animals.

Table 2 shows the effect of bile volume of bromsulphthalein in the two cats, 8 and 9. It can be seen that, during the control period of 60 or 80 minutes, the bile volume in mls. per minute is respectively 0.0354 and 0.030 mls. per minute. During the 60 minutes following the injection of bromsulphthalein, the bile volume is increased in cat 8 by approximately 25.7% and in cat 9 by 59.3%. As time proceeds, it can be seen that in both these animals, the precentage increase in bile volume decreases as the choleretic effect of bromsulphthalein wears off. In the second part of this table, is given the amount of bromsulphthalein recovered at various times in the bile. The total quantity of bromsulphthalein recovered after a particular period of time, as well as the individual percentage of dye recovered, is also given in tabular form. In both these cases, it is to be noted, there is only a small percentage of the dose given recovered in the first 10 minutes, as has been mentioned previously.

Table 2.

The first part of the Table shows the effect of bromsulphthalein on the bile volume in the acute biliary fistula cats, 8 and 9. It can be seen that there is a definite choleretic effect. The second part of the Table details (i) the amount of dye recovered over a given time period; (ii) the total amount of dye recovered at the end of any particular collecting period; and (iii) the percentage of the dose given which has been recovered as time proceeds in the bile of cats 8 and 9.

Cat 8 Dose of Bromsulphthalein 5 mg/Kg body weight (15.5 mg. given)

Bile Volume

Control Volume = 0.0354 ml/min. Control Period = 80 minutes
Period after Bromsulphthalein (0 - 60 minutes) = 0.0445 (+ 25.7%)
" " " (0 - 110 ") = 0.0389 (+ 9.9%)

BROMSULPHTHALEIN in BILE

Time (Minutes)	Amount of dye (mg)	Total	% recovered
0 - 20	1.02	1.02	6.61
20 - 30	2.74	3.76	24.3
30 - 41	3.04	6.80	44.1
41 - 50	1.69	8.49	54.8
50 - 60	1.04	9.54	61.4
60 - 70	0.69	10.23	66.1
70 - 81	0.49	10.72	69.4
81 - 90	0.21	10.93	70.6
90 - 110	0.25	11.18	72.1

In the first 10 minutes, only 0.009 mgm. of Bromsulphthalein was recovered, that is 0.058% of the dose given.

Cat 9 Dose of Bromsulphthalein 5 mg/Kg body weight (16.5 mg.given)

Bile Volume

Control Volume - 0.0300 ml/min. Control Period - 60 minutes
Period after Bromsulphthalein (0 - 60 minutes) - 0.0478 (+59.3%)
" " " (0 - 135 ") - 0.0418 (+39.3%)

BROMSULPHTHALEIN in BILE

Time (Minutes)	Amount of dye (mg)	Total	% recovered
0 - 21	1.30	1.30	7.9
21 - 30	2.74	4.04	24.5
30 - 40	3.04	7.08	42.9
40 - 50	1.79	8.87	53.7
50 - 60	0.88	9.75	59.0
60 - 75	0.57	10.31	62.6
75 - 90	0.24	10.55	64.0
90 - 120	0.17	10.72	65.0
120 - 130	0.07	10.80	65.5

In the first 10 minutes, only 0.055 mgm. of Bromsulphthalein was recovered, that is 0.333% of the dose given.

If a plasma volume of 50 mls./Kg. body weight is accepted and a 5 mg./Kg. dose of bromsulphthalein given, then, if instant mixing of the dye occurred following an intravenous injection (which it does not), the initial blood concentration will be 10 mg./100 mls. Table 3 takes advantage of this fact in determining the percentage of the dose which has been given which remains in the plasma at ten minutes. The six normal animals, cats 1 - 7, cat 4 having been excluded because of the opalescence of the plasma following an unexpected meal, as previously mentioned, have an average precentage retention of 5% at 10 minutes. From the Table 2 and Figures 26 and 27, we know that the recovery of dye at 10 minutes is very small and therefore it seems reasonable to accept that at 10 minutes there is approximately 95% or over of the dye injected present in the liver. In order to estimate the dye recovery from the liver as a terminal event, it has been assumed that 5% of the dose of bromsulphthalein given remains in the bile or in the blood. In the five cats (11 - 15 inclusive) a 20 mg. dose of bromsulphthalein was injected and the animals killed at 10 minutes. The liver was immediately removed and its dye content estimated using the ex-

Table 3

This table details the percentage of the zero time concentration of bromsulphthalein remaining in the plasma 10 minutes after a 5 mg./Kg. body weight dose given intravenously. It was assumed that the zero time concentration is 10 mg./100 ml. with a plasma volume of 50 ml./Kg. body weight and a dose of bromsulphthalein 5 mg./Kg. body weight.

6 Normal Intact Cats

Cat No.	Time	% of dose given remaining in Plasma
1	10 mins.	5.2
2	10 mins.	3.2
3	10 mins.	5.1
5	10 mins.	6.4
6	10 mins.	2.5
7	10 mins.	3.6

Average % of dose given remaining in Plasma = 5.1%

Cat 4 had just eaten and the plasma was opalescent so could not be estimated properly in the Absorptionmeter.

traction process described in the methods.

Table 4 gives the actual recovery of dye present in the various portions of the liver examined. The expected content of dye in these portions has been based on a uniform distribution of bromsulphthalein within the liver. Four or five specimens of liver have been examined in each cat so that the whole of the liver was examined. At the same time, certain of the specimens were examined fresh, i.e. shortly after the animal had been killed, some were examined after 24 hours after being kept in the refrigerator overnight and others were examined after a period of 7 days, the portion of liver, meanwhile, being kept in the refrigerator. The main purpose for the examination at different times was to see whether or not the recovery was similar after a different time interval. Another reason was that it was virtually impossible to carry out all the estimations immediately, since each extraction took an average of 3 - 4 hours. It can be seen that only a portion of the expected content of dye was recovered, in fact, approximately 50%. In the Tables, the conversion factor and percentage recovery in the individual cats is also given.

Table 4

5 cats (numbers 11 - 15 inclusive) have been given 20 mg. of bromsulphthalein intravenously and killed 10 minutes later. At this time it is anticipated that in the 'normal' animal there would be at least 95% of the dye injected within the liver. This means that 5% of the dose given (1 mg.) is present within the circulation, bile or urine. This would appear to be a reasonable estimate since, in each cat, the amount of dye in the terminal blood specimen was always less than 0.5 mg., the urine contained no bromsulphthalein and it has been shown that the amount of dye present in the extra-hepatic biliary tree and cannula is virtually zero.

The individual recoveries in fresh specimens, 24 hours old and 7 day old specimens is compared in the individual cats.

If a dose of 20 mgm. Bromsulphthalein is given and 5% of the dose remains in the blood, bile or urine at 10 minutes, 19 mgm. of Bromsulphthalein should therefore be present in the liver. Assuming there is uniform distribution of dye within the liver the expected content of dye can be estimated in each portion of liver examined. All cats given 20 mgm. Bromsulphthalein.

Weight of Liver Portion (gm)	Time of Examination	Expected Content (mg)	Brom-sulphthalein Recovered (mg)	Factor	% Recovery
	Cat 11 (Weight of Liver - 94 gm.)				
20	Fresh	4.04	2.10	1.92	52
20	Fresh	4.04	1.98	2.04	49
20	Fresh	4.04	2.05	1.97	50.9
20	24 hours	4.04	1.96	2.12	48.6
14	7 days	2.84	1.40	2.03	49.4
	Cat 12 (Weight of Liver - 82.3 gm.)				
20	Fresh	4.63	2.30	2.02	49.7
20	Fresh	4.63	2.52	1.84	54.5
20	24 hours	4.63	2.44	1.90	52.7
22.3	7 days	5.11	2.62	1.95	51.2
	Cat 13 (Weight of Liver - 74 gm.)				
20	Fresh	5.14	2.58	1.99	50.3
20	Fresh	5.14	2.40	2.14	47.8
20	24 hours	5.14	2.65	1.94	51.7
14	7 days	3.58	1.90	1.89	53.1

Weight of Liver Portion (gm)	Time of Examination	Expected Content (mg)	Brom-sulphthalein Recovered (mg)	Factor	% Recovery
	Cat 14 (Weight of Liver - 72.8 gm.)				
20	Fresh	5.22	2.80	1.87	53.7
20	Fresh	5.22	2.55	2.05	48.9
20	24 hours	5.22	2.60	2.01	49.8
12.8	7 days	3.34	1.50	2.23	45.0
	Cat 15 (Weight of Liver - 88.7 gm.)				
20	Fresh	4.28	2.20	1.95	51.4
20	Fresh	4.28	2.08	2.06	48.7
20	24 hours	4.28	2.30	1.87	53.8
28.7	7 days	6.16	2.80	2.19	45.4

Table 5 gives a summary of the amounts of dye recovered. Those which were examined after 24 hours, 51.3% was recovered and after 7 days 48.7% of the dye given was recovered. The expected dye content given was recovered. There would thus appear to be a reasonable degree of agreement between these results, allowing for experimental errors. The total amount of dye given to these animals was 100 mg. and the anticipated content of liver at 10 minutes was 95 mg. The actual amount recovered was 47.73 mg. and the percentage recovery was 50.3%, which gives a conversion factor of 1.99.

No attempt to determine the dye content of bromsulphthalein in any other compartment of the cat was carried out, apart from the experiments on cats 11 to 15 invlusive, in which the urine was collected from the bladder and analysed for bromsulphthalein. In none of these 5 cats was there any detectable bromsulphthalein to be found in the urine at 10 minutes. The terminal blood samples in each of the five cats (11 - 15 inclusive) was 0.5 mg. or less.

Table 5

This table compares the actual amount recovered and percentage recovery of bromsulphthalein from cat liver when examined, fresh, after 24 hours and 7 days respectively. The total recovery is given as well and a conversion factor calculated.

AMOUNT OF DYE RECOVERED FROM CAT LIVER

	Expected Content (mgs.)	Amount Recovered (mgs.)	%
Specimens examined Fresh	50.66	25.56	50.6
Specimens examined after 24 hours	23.3	11.95	51.3
Specimens examined after 7 days	21.03	10.22	48.7
Total	95.00	47.73	50.3

$$\therefore \text{ Conversion Factor} = \frac{95.00}{47.73} = 1.99$$

Summary

Preliminary experiments were carried out on rats and cats in order to obtain some information about the temporal distribution of bromsulphthalein within the blood, liver and bile compartments. It was also the purpose of this series of experiments to assess the usefulness of the method devised for the recovery of bromsulphthalein from the liver. The extraction process described in the Methods, yielded a satisfactory and consistent proportion of the expected dye content of the liver in cats 11 - 15 inclusive. Although accepted, there are other possible sites for bromsulphthalein in the body (see Figure 41). It has been assumed that all the dye which cannot be located in the bile or blood is within the "liver". The assumed "liver content" of dye is, in fact, that present in the liver and any other tissue(s) or site(s) which may take up bromsulphthalein. In these preliminary experiments, there was no evidence of a renal excretion of dye and the blood content was of such low order that it seems unlikely that there could be a great loss of bromsulphthalein to other tissues. As previously

mentioned, in the literature which has been reviewed, in the normal 'intact' animal, the liver is the principal site for the extraction of dye from the circulation. Even in cat 10, who was given an extremely large dose of bromsulphthalein, 63 mg./Kg. body weight, the dye is still removed reasonably efficiently from the circulation, since two-thirds of the initial concentration has disappeared in almost forty minutes. Although not confirmed, since bile was not collected in this cat, it would appear that the maximum excretory capacity of the liver had, in fact, not been exceeded.

THE TEMPORAL DISTRIBUTION OF BROMSULPHTHALEIN IN THE DOG

Hepatic Extraction of Bromsulphthalein

In order to show that the liver extracts a considerable quantity of bromsulphthalein from the circulation, three dogs were anaesthetised and a cardiac catheter inserted via the anterior jugular vein into the right side of the heart and then passed through the inferior vena cava into one of the main hepatic veins, being visualised on an X-ray screen. Serial blood samples were then collected at frequent

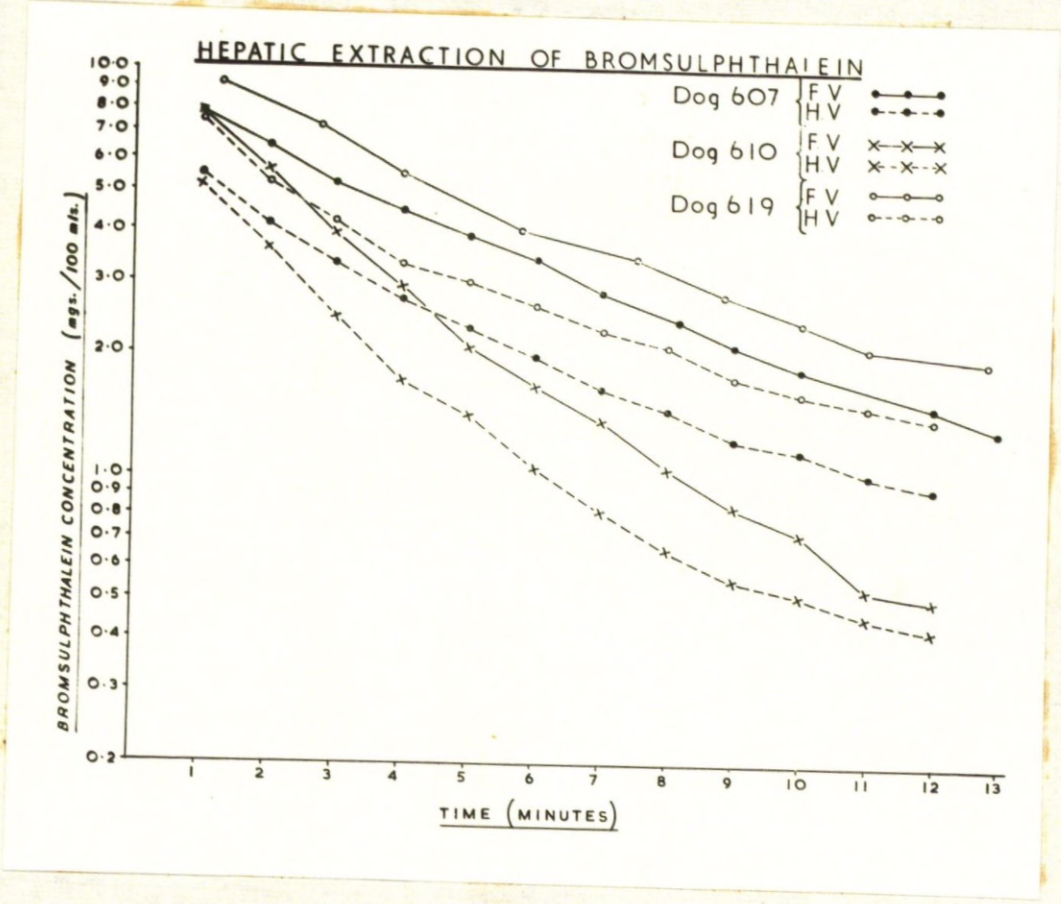
intervals over a period of twelve to thirteen minutes from the femoral vein and the hepatic vein. The results in these three experiments in which the dogs were given a 5 mg./Kg. dose of bromsulphthalein is shown in Figure 29. This Figure shows the bromsulphthalein plasma concentration in mg./100 mls. plotted against time. It can be seen that the peripheral blood concentrations fall approximately on a straight line in each case and the hepatic vein samples also fall on straight lines which closely parallel the peripheral blood concentration of the particular animal concerned. In dog 607, for example, at six minutes, the peripheral blood concentration is 3.47, whereas the concentration in the hepatic vein is 1.98. This means that almost 45% of the dye has been removed.

In fact, however, it would be expected to be greater, since the six-minute hepatic vein concentration will correspond to a time somewhat prior to six minutes in blood reaching the liver. The peripheral venous blood concentration of bromsulphthalein reflects the arterial concentration of dye. Since the liver receives its blood supply from the hepatic artery and portal venous system, the peripheral venous blood is an estimate of the concentration of dye reaching the

Figure 29

The hepatic extraction of bromsulphthalein

The femoral vein plasma concentration of dye in dogs 607, 610 and 619 is compared with the hepatic vein plasma concentration of dye. In each case, a 5 mg/Kg. body weight dose of bromsulphthalein was given intravenously and the samples of blood taken at minute or two-minute intervals for a period of 12 - 13 minutes after the injection. The hepatic vein blood was obtained from a cardiac catheter after it had been inserted through the heart into a hepatic vein under direct X-ray screening.


HEPATIC EXTRACTION OF BROMSULPHTHALEIN
Dog 607
F V
H V
Dog 610
F V
H V
Dog 619
F V
H V
BROMSULPHTHALEIN CONCENTRATION (mgs./100 mls.)
10·0
9·0
8·0
7·0
6·0
5·0
4·0
3·0
2·0
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
1
2
3
4
5
6
7
8
9
10
11
12
13
TIME (MINUTES)

liver. The hepatic vein sample which has been taken is the concentration existing in the particular hepatic vein catheterised. Because the catheter was visually situated fairly deep in the liver substance, it thus reduced the likelihood of much regurgitation of blood from the inferior vena cava into the catheterised branch of the hepatic vein. Although it is not strictly possible to compare any two individual femoral and hepatic concentrations, at similar times, it does seem that over the given twelve or thirteen minutes a considerable proportion of the dye presented to the liver is extracted from the blood flowing through the liver.

The Entero-Hepatic Circulation

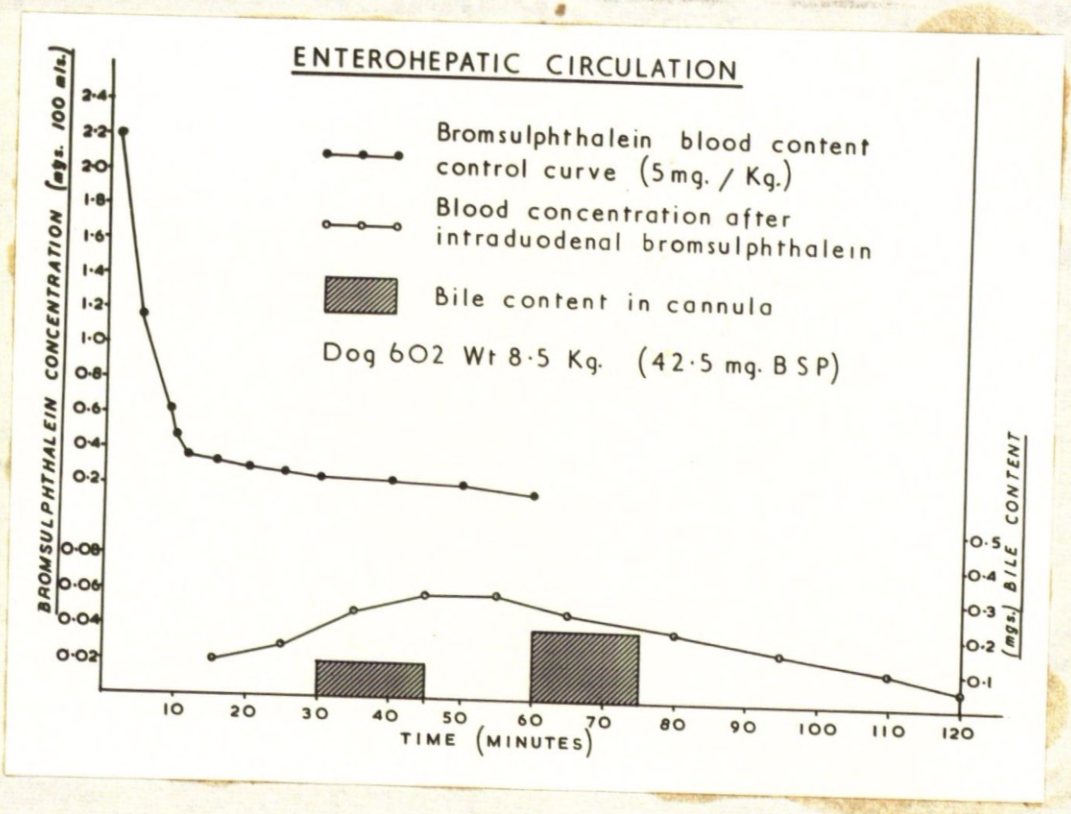
Figure 30 shows an attempt to estimate the significance of the entero-hepatic circulation of bromsulphthalein in dogs. In this particular animal, dog 602, a 5 mg. dose of bromsulphthalein was given and the blood concentration of dye estimated for a period of sixty minutes. The plasma concentrations are plotted against time as shown in the Figure. It can be seen that after twelve minutes, the concentration is consistently below 0.4 mg.% and just below 0.2 mg.% at sixty minutes. Two weeks later,

Figure 30

The entero-hepatic circulation of bromsulphthalein

A normal disappearance curve of bromsulphthalein from the plasma after a 5 mg./Kg. body weight dose is compared with the plasma concentrations obtained two weeks later, following the intra-duodenal administration of an identical dose (42.5 mg.) of bromsulphthalein.

The amount of dye recovered in the bile is also given.


ENTEROHEPATIC CIRCULATION
Bromsulphthalein blood content control curve (5 mg. / Kg.)
Blood concentration after intraduodenal bromsulphthalein
Bile content in cannula
Dog 602 Wt 8·5 Kg. (42·5 mg. BSP)
BROMSULPHTHALEIN CONCENTRATION (mgs. 100 c/cs.)
2·4
2·2
2·0
1·8
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0·08
0·06
0·04
0·02
0·5
0·4
0·3
0·2
0·1
(mgs.) BILE CONTENT
10
20
30
40
50
60
70
80
90
100
110
120
TIME (MINUTES)

the same dose of bromsulphthalein (i.e. 42.5 mg. of bromsulphthalein) was given intra-duodenally and the blood concentrations observed over a period of two hours. Prior to the intra-duodenal injection of bromsulphthalein an acute biliary fistula had been performed, so that bile could be collected at fifteen minute intervals and examined for bromsulphthalein. It can be seen that, under the conditions of this experiment, in which there was no bromsulphthalein at zero time remaining in the plasma from the previous intravenous injection two weeks earlier, the blood concentrations were of a very low order. The highest concentration reached being 0.06 mg./100 mls. and this occurring forty-five to fifty-five minutes after the intra-duodenal administration of bromsulphthalein. These concentrations are indeed very low and, in fact, are the lower limits of accuracy using colorimetric methods for the determination of bromsulphthalein. On only two occasions during the fifteen minute periods analysed over a period of two hours, was any bromsulphthalein present in the bile. In the fifteen minute period from thirty to forty-five minutes, there was a total of 0.1 mg. of brom-

sulphthalein recovered and in the sixty to seventy-five minute period, 0.2 mg. of bromsulphthalein was recovered. Following a standard intravenous injection of 5 mg./Kg. of bromsulphthalein it can be anticipated that the amount of bromsulphthalein that will be present in the duodenum for the entero-hepatic circulation to occur, will always be much lower than 5 mg./Kg., which was the quantity injected on this occasion. It would therefore appear that the entero-hepatic circulation is negligible, particularly in the usual time period of observation (60 minutes). Under normal circumstances, there is a delay in the appearance of bromsulphthalein into the bile and therefore the inevitable delay in the entero-hepatic circulation would mean that any quantity of dye re-entering the circulation would, in effect, probably be occurring after sixty minutes. With larger doses of bromsulphthalein and longer experiments it is possible that the entero-hepatic circulation however, may be a definite factor. It is of interest to note that even when 5 mg./Kg. of bromsulphthalein was injected intra-duodenally in this experiment, there would at no time be more, if as much, as 0.5 mg./100 mls. of bromsulphthalein. In the experiment

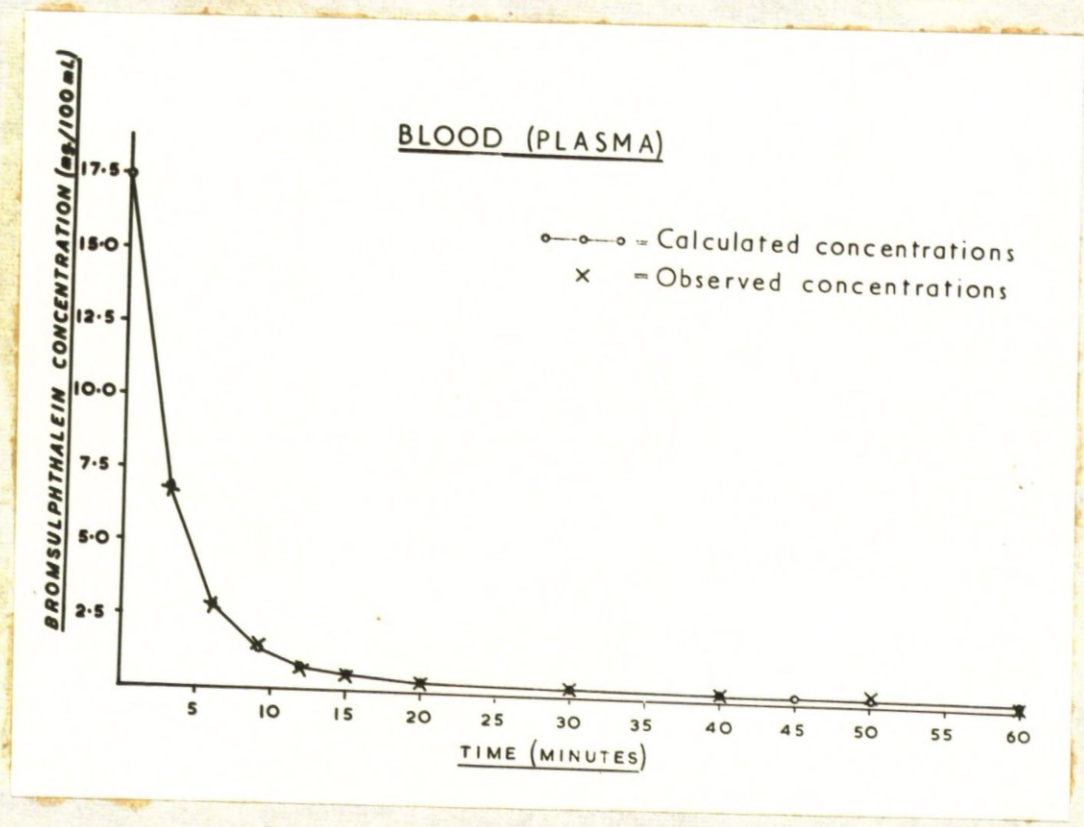
detailed in Figure 30, the estimated plasma volume in Dog 602 would be 425 mls. and the highest blood content of dye would be of the order of 0.3 mg., which is less than 1% of the dose given.

The Distribution of Bromsulphthalein and the Mathematical Model.

A mathematical model has been derived by Mr. Andrew Young (Department of Applied Mathematics, University of Liverpool), and the mathematical derivation of this model is given separately in the Appendix at the end of this Section. The mathematical model is based on bromsulphthalein being present in the blood, liver or bile and the figures which have been derived from this mathematical model are based on the observed plasma concentrations of bromsulphthalein alone. In the Figures and Tables in this section, the values for the calculated concentrations or content of dye are those which have been obtained mathematically with the aid of an Electronic Computer. Figure 31 shows the calculated concentration of bromsulphthalein compared with the observed concentrations. As would be expected, the calculated and the observed concentrations correspond

Figure 31

The observed plasma concentrations compared with those calculated using the mathematical model. The calculated concentrations are, in fact, based on the "best fit" of the observed concentrations, using the method of least squares.


BLOOD (PLASMA)
o—o—o = Calculated concentrations
× = Observed concentrations
BROMSULPHTHALEIN CONCENTRATION (mg/100 ml)
17·5
15·0
12·5
10·0
7·5
5·0
2·5
5
10
15
20
25
30
35
40
45
50
55
60
TIME (MINUTES)

closely, since the calculated concentrations derived mathematically are, in fact, based on these observed concentrations. When the logarithm of the plasma concentrations is plotted against time, it can be seen that the calculated plasma concentration can be represented graphically by the sum of two separate lines. This is shown in Figure 32. Once again, it can be seen that there is a close correlation between the observed calculations and those calculated. At any given time, the concentrations can be derived from the equation $Cx = Ae^{-k_1 t} + Be^{-k_2 t}$ in which A and B are constants as indicated on the graph, k_1 and k_2 are the slopes of the individual component lines and t is the time. The two component lines are represented by the equation $Cx_1 = Ae^{-k_1 t}$ and $Cx_2 = Be^{-k_2 t}$.
It will be seen that there is a "bend" in the graph as the change from one line to the other occurs. So that the earlier points approximate to the slope of the first line and the later points correspond more closely to the slope of the second phase. The initial phase appears to take place rapidly, whereas the later phase takes place more slowly. These equations, as explained in the mathematical Appendix, form the basis of the mathematical model.

Figure 32

The logarithm plasma concentration plotted against time. The calculated concentration is plotted and the observed concentrations given separately. The graph can be represented mathematically by the equation $C_x = A_e^{-k_1 t} - Be^{-k_2 t}$ which consists of two component lines $C_{x_1} = Ae^{-k_1 t}$ and $C_{x_2} = Be^{-k_2 t}$ as indicated in the graph. The "bend" represents the change from the initial rapid phase to the second slower phase. The mathematical model is based on this equation as indicated in the Appendix. The derivation of the constants A and B are given in the Figure.

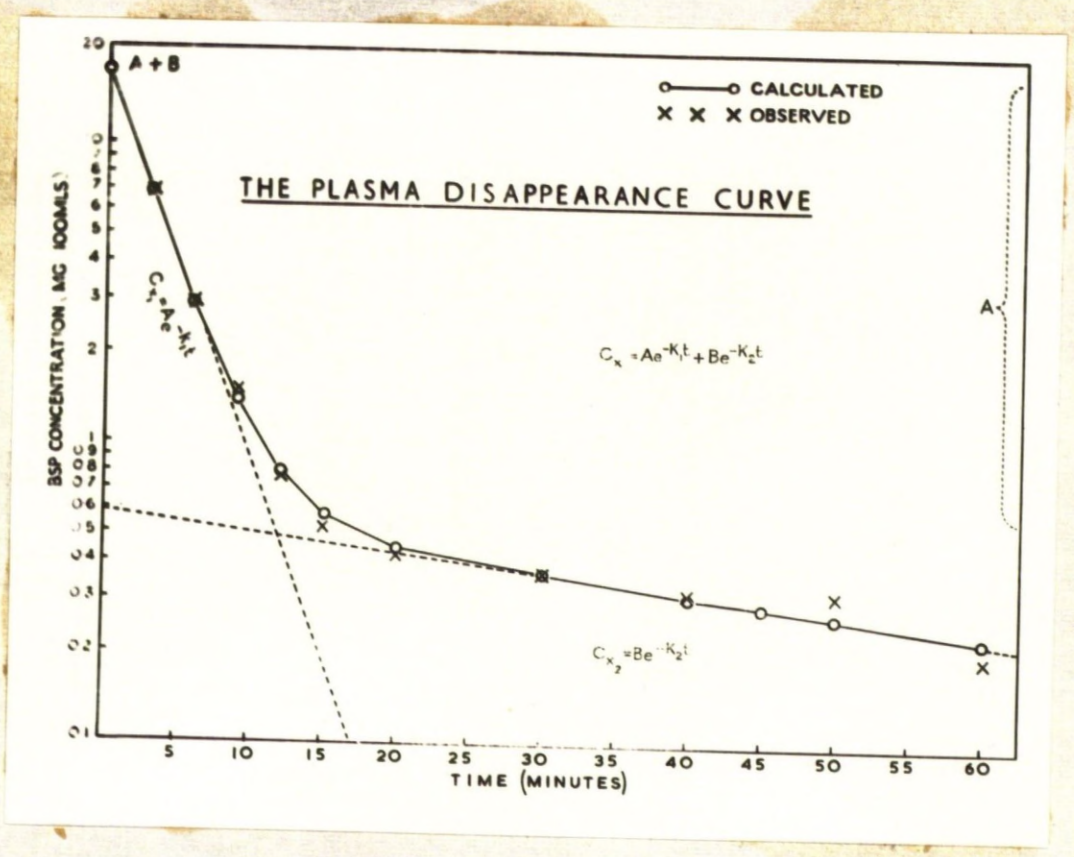

THE PLASMA DISAPPEARANCE CURVE
CALCULATED
OBSERVED
A+B
A
$C_x = Ae^{-K_1 t} + Be^{-K_2 t}$
$C_{x_1} = Ae^{-K_1 t}$
$C_{x_2} = Be^{-K_2 t}$
BSP CONCENTRATION (MG %OMLS)
TIME (MINUTES)
20
10
5
2
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
5
10
15
20
25
30
35
40
45
50
55
60

Figure 33 shows a comparison of the observed and calculated content of dye present in the bile. The calculated figures have been derived from the plasma concentrations alone, using the mathematical model. It can be seen in this particular experiment in which the dog was given a dose of 80 mg. (5 mg./ Kg. body weight) there is a lag between the observed and calculated content of bromsulphthalein. This is due to the inevitable dead space present in the biliary system within the liver and outside the liver in the bile ducts, as well as in the polythene cannula which has been used. It can be seen that at the end of sixty minutes, when the observed recovery of dye is taken plus the amount of dye recovered from the cannula, and is compared with the amount of bromsulphthalein calculated, there is a close correlation between the respective values. In the experiments which have lasted over sixty minutes, the approximation between the observed and calculated content of dye has been closer the longer the experiment continues.

Figure 34 shows the calculated blood content of dye compared with the observed and calculated bile recovery of dye. Mathematically, it is easier to determine the blood content of dye, since the assumption

Figure 33

A comparison of the observed and calculated content of dye in the bile. The observed content of dye lags behind that calculated but as time proceeds it approximates more closely to the calculated quantity.
The terminal content of dye recovered, includes the dye in the dead space of the cannula and is given as a separate point. When the dye in the cannula is added to the observed recovery there is very close agreement to the calculated value present at 60 minutes.

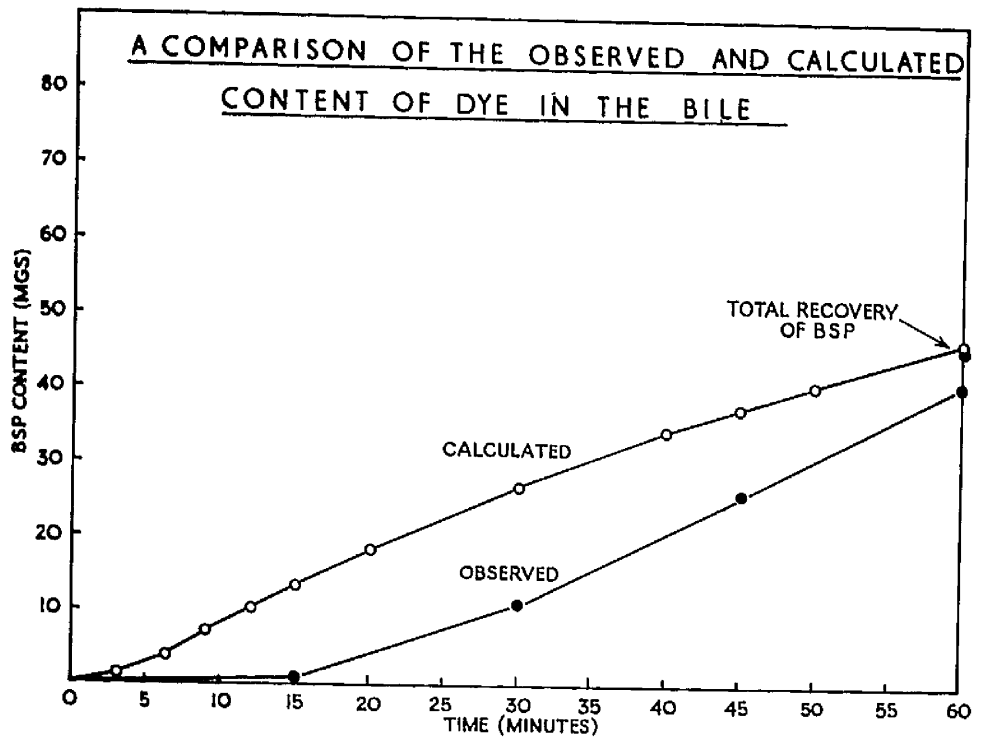
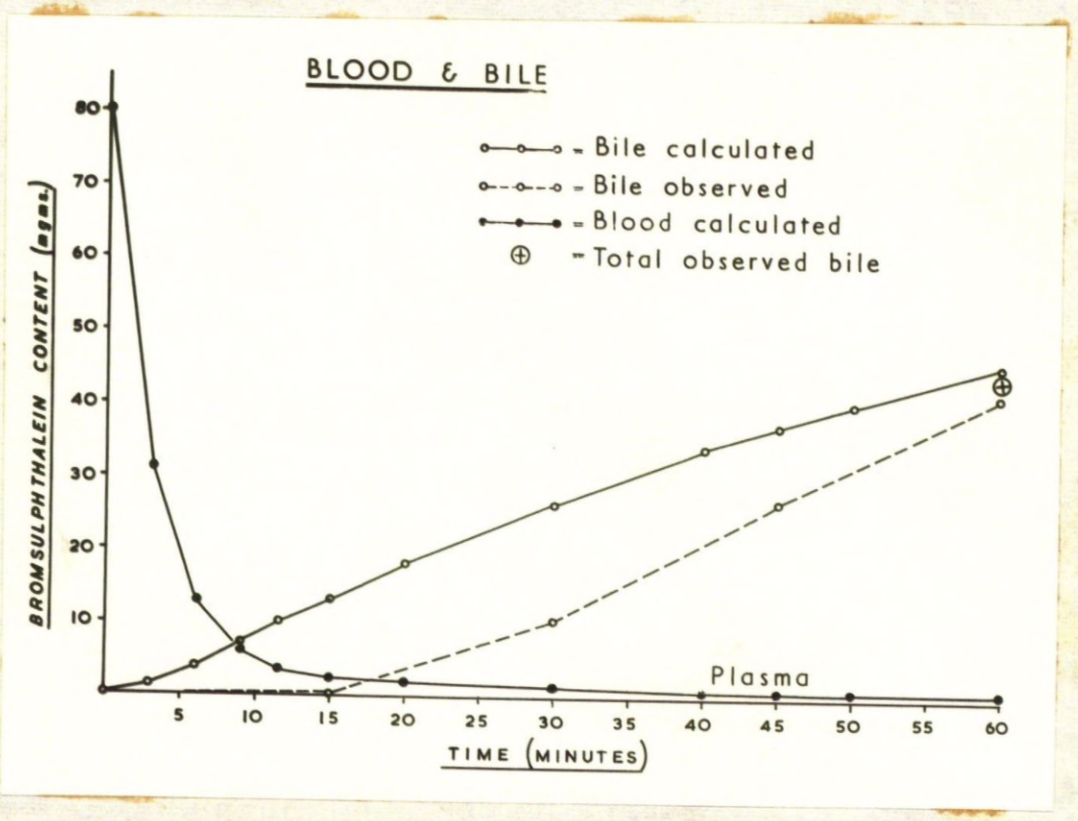

A COMPARISON OF THE OBSERVED AND CALCULATED
CONTENT OF DYE IN THE BILE
BSP CONTENT (MGS)
80
70
60
50
40
30
20
10
0
5
10
15
20
25
30
35
40
45
50
55
60
TIME (MINUTES)
TOTAL RECOVERY OF BSP
CALCULATED
OBSERVED

Figure 34

A comparison of the observed and calculated content of dye in the bile. The observed content of dye lags behind that calculated but as time proceeds it approximates more closely to the calculated quantity. The terminal content of dye recovered includes the dye in the dead space of the cannula and is given as a separate point, which is very close to the value calculated to be present at 60 minutes. Also given is the calculated blood content of bromsulphthalein in order to show the distribution of dye in the blood and bile compartments.

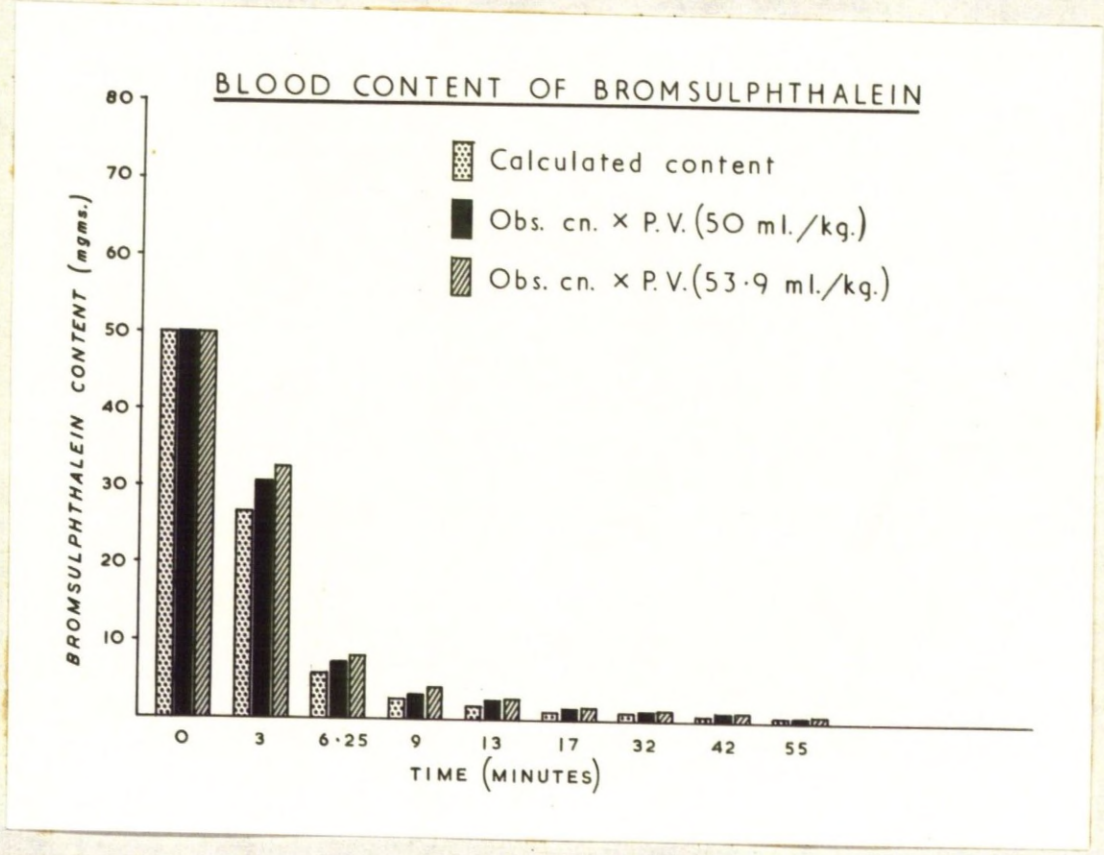

BLOOD & BILE
Bile calculated
Bile observed
Blood calculated
Total observed bile
BROMSULPHTHALEIN CONTENT (mgms.)
80
70
60
50
40
30
20
10
Plasma
5
10
15
20
25
30
35
40
45
50
55
60
TIME (MINUTES)

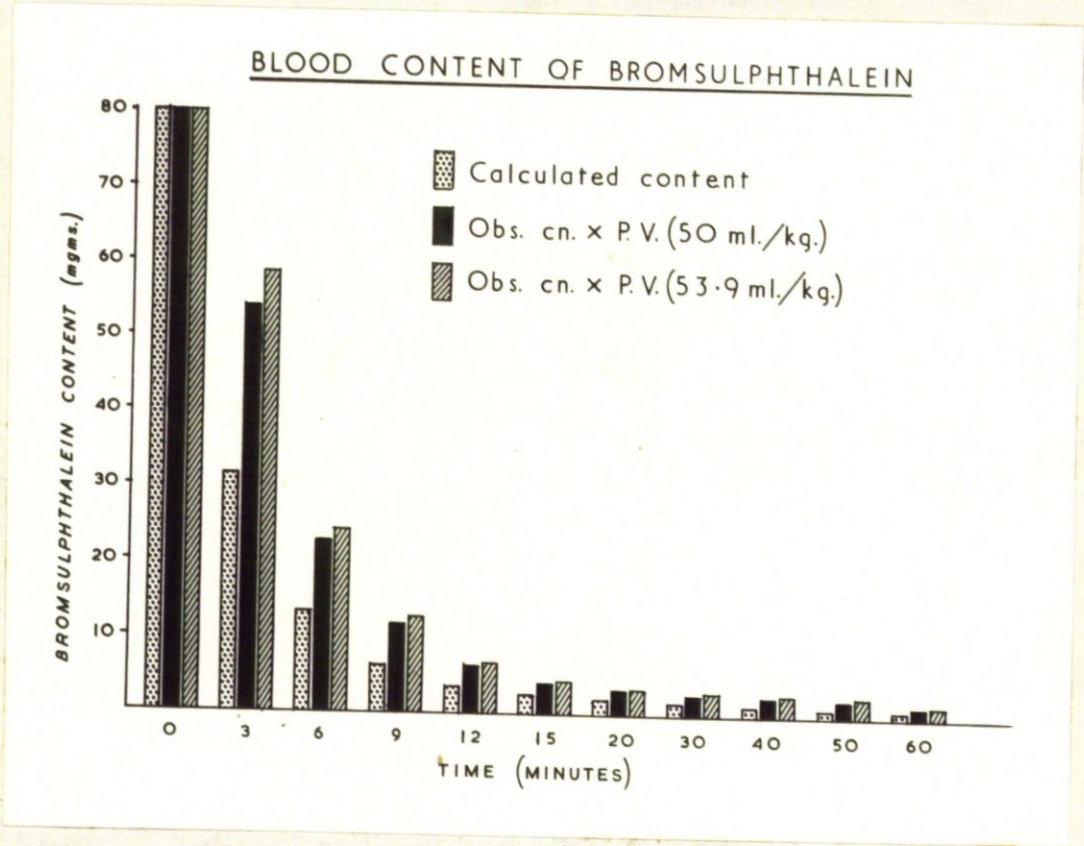
that initially all the bromsulphthalein must be in the blood, means an estimate of the so-called plasma volume is given by the initial concentration. Though, due to the rapid diffusion of bromsulphthalein from the circulatory compartment, it is obvious that this will only give a value of the compartment in which the bromsulphthalein is contained and not the true plasma volume. The blood content of dye in the dog experiments has been based entirely on a plasma volume of 50 mls./Kg. body weight, although this is not strictly accurate in dogs, since Gibson, Peacock, Seligman and Sack (1946) have found that the plasma volume in normal mongrel dogs closely corresponds to a volume of 53.9 mls./Kg. body weight. Bromsulphthalein combines with the plasma proteins in the blood and therefore the plasma concentration of bromsulphthalein is equivalent to the blood concentration of dye.

Figures 35 and 36 give a comparison between the blood content of bromsulphthalein in two dogs, in which 50 and 80 mg. of bromsulphthalein has been given respectively. A comparison is made between the calculated blood content of dye with the "observed content", obtained by multiplying the observed

Figures 35 and 36

A comparison of the blood content of bromsulphthalein in dogs 1 and 2. The calculated content of dye derived from the mathematical model is compared with that obtained by multiplying the observed plasma concentration by an accepted plasma volume based on body weight of 50 mls./Kg. and 53.9 mls./Kg. respectively.


BLOOD CONTENT OF BROMSULPHTHALEIN
Calculated content
Obs. cn. × P.V. (50 ml./kg.)
Obs. cn. × P.V. (53·9 ml./kg.)
BROMSULPHTHALEIN CONTENT (mgms.)
80
70
60
50
40
30
20
10
0
3
6·25
9
13
17
32
42
55
TIME (MINUTES)


BLOOD CONTENT OF BROMSULPHTHALEIN
Calculated content
Obs. cn. × P.V. (50 ml./kg.)
Obs. cn. × P.V. (53·9 ml./kg.)
BROMSULPHTHALEIN CONTENT (mgms.)
80
70
60
50
40
30
20
10
0
3
6
9
12
15
20
30
40
50
60
TIME (MINUTES)

concentrations by a plasma volume of 50 mls./Kg. and 53.9 mls./Kg. respectively. It can be seen that there is a difference between the observed blood content derived in this manner and the calculated content. As the dye is removed from the blood compartment, the figures from the individual methods reveal the difference between the blood contents more markedly, although after 15 minutes the actual amount of bromsulphthalein concerned is small, in relation to the dose given.

The mathematical model can give a value for the liver content of dye at any given time, but it is only possible to determine the terminal content of bromsulphthalein as a single reading. A graphical representation of the mathematical model is given in Figure 37. This figure shows the dye content of blood, liver and bile in relation to time, and the two-way transfer of dye between the blood and liver, and the one-way excretion of bromsulphthalein into the bile compartment is indicated by small symbols in the figure. The model is, in fact, based on three factors concerned: (i) the transfer of dye from the blood to the liver, (ii) the transfer of dye from the liver to the blood and (iii) the transfer of dye from the liver to the bile.

Figure 37

The temporal distribution in dog 2 of bromsulphthalein, according to the mathematical model. This accepts that injected bromsulphthalein exists only within the blood, liver and bile compartments and that an exchange of dye takes place between the blood and liver, the liver and blood and the liver and bile (as indicated at the top of the figure)

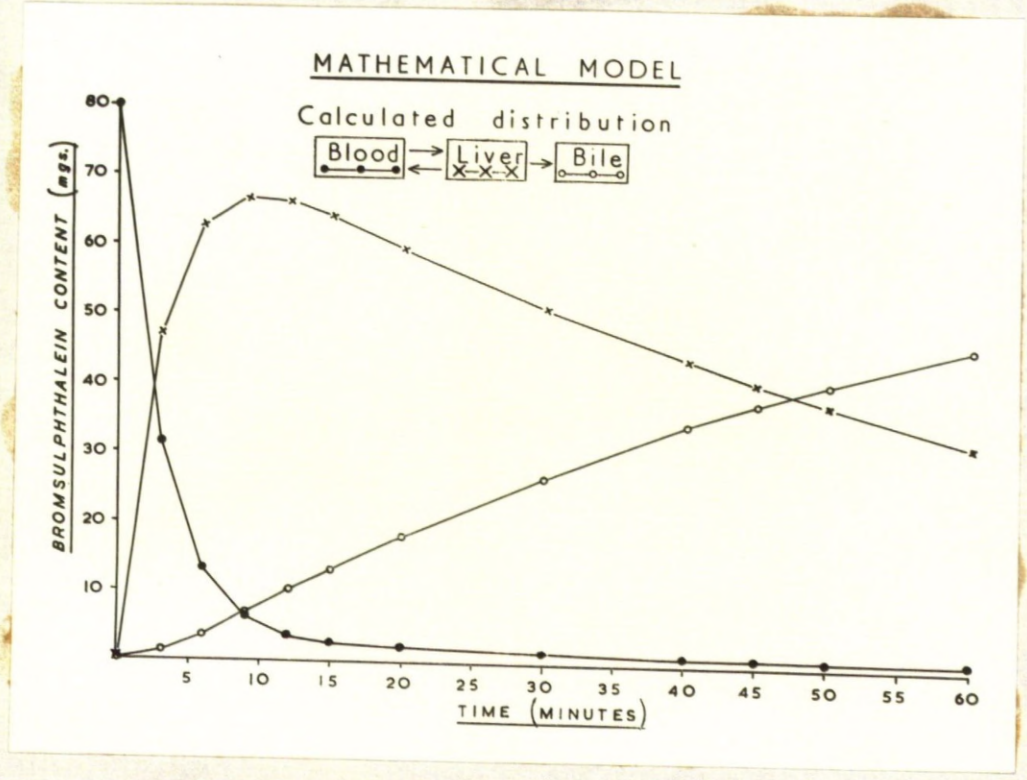

MATHEMATICAL MODEL
Calculated distribution
Blood
Liver
Bile
BROMSULPHTHALEIN CONTENT (mgs.)
80
70
60
50
40
30
20
10
5
10
15
20
25
30
35
40
45
50
55
60
TIME (MINUTES)

Figure 38 shows a comparison of the mathematical model with the observed distribution of dye. The blood content has been derived as mentioned previously and the observed bile recovery, including the dead space, bromsulphthalein is given. The "observed liver content" has been based entirely on the assumption that the quantity of dye which is not present in the blood and bile is in the 'liver'. It can be seen that there is a reasonable correlation between the observed and calculated distribution and there exists a similar pattern in the distribution of the dye. Since the results are similar in the series of 9 normal animals investigated for the temporal distribution of bromsulphthalein, in this section only one has been depicted graphically. The results, which have been obtained in the nine animals have, however, been given in Table 6, but the terminal content of bromsulphthalein recovered from the blood, liver and bile are compared.

In this Table, the duration of each experiment is given, with the exception of dog 3 the duration has been 60 minutes. The dose at all times has been 5 mg./Kg. body weight. The comparison of the observed and calculated terminal blood contents would

Figure 38

A comparison of the "observed content" of dye in the blood, bile and liver with the calculated content derived using the mathematical model. The observed blood content is based on a plasma volume of 50 mls./Kg. body weight, the bile content takes into account the dye in the cannula at the end of 60 minutes, the "observed liver content" is the amount of dye which is not present in the blood and bile compartments.

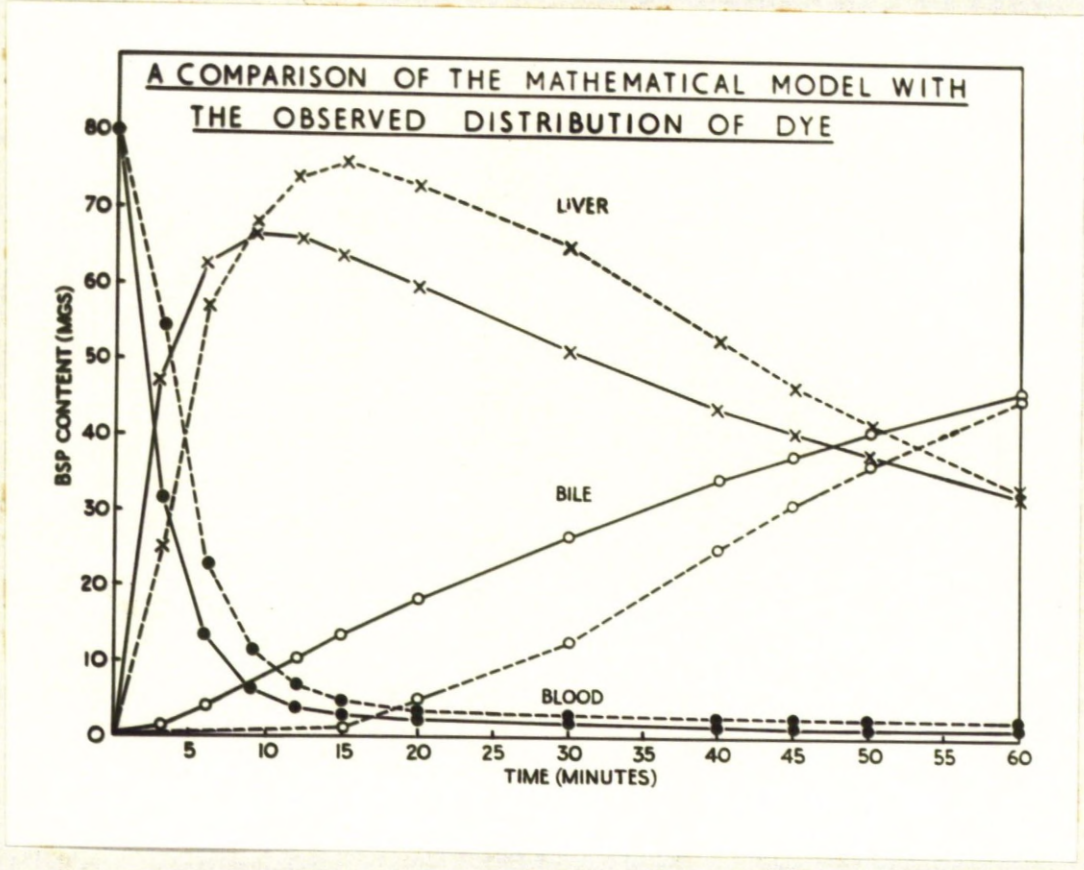

A COMPARISON OF THE MATHEMATICAL MODEL WITH
THE OBSERVED DISTRIBUTION OF DYE
BSP CONTENT (MGS)
0
10
20
30
40
50
60
70
80
LIVER
BILE
BLOOD
5
10
15
20
25
30
35
40
45
50
55
60
TIME (MINUTES)

Table 6

This table gives the terminal content of bromsulphthalein in the blood, bile and liver in the 9 normal acute biliary fistula dogs. The observed terminal quantity of dye in the various sites is compared with that calculated from the mathematical model based on the observed plasma concentrations alone. The dose of dye and the duration of each experiment is given. The total amount of dye found in the urine for the whole of the time period of the experiment is also given.

An "abnormal" dog (10) is compared with the 'normal' dogs.

Dog number	Duration of Experiment (mins.)	Dose of Bromsulphthalein (5 mg/Kg)	Terminal Blood Content (mg)		Terminal Bile Content (mg)		Terminal Liver Content (mg)			Total content of Bromsulphthalein in urine (mg)
			Observed	Calculated	Observed	Calculated	Observed	Range for Observed Recovery	Calculated	
1	60	50	0.92	1.05	31.3	29.3	7.4	18.5 - 24.7	19.42	0.60
2	60	80	1.72	0.65	45.9	46.53	12.3	30.8 - 41.0	32.42	0.66
3	120	100	1.61	0.46	75.45	80.77	5.3	13.3 - 17.7	18.77	0.45
4	60	50	1.94	1.15	21.85	24.71	7.0	17.5 - 23.3	23.14	0.40
5	60	42.5	0.60	0.83	24.92	29.9	3.5	8.75- 11.65	11.77	0.25
6	60	90	0.97	0.69	54.12	60.51	9.5	23.68- 30.03	28.8	0.75
7	60	70	2.72	1.74	36.5	40.18	8.7	21.74- 28.70	28.08	0.68
8	60	35	1.17	1.06	9.68	9.88	7.7	19.3 - 25.7	24.06	0.36
9	60	50	1.51	1.12	23.87	28.26	8.3	20.8 - 27.7	20.62	0.48
10	60	75	4.95	3.24	10.41	14.76	19.2	48.0 - 64.0	57.0	1.30

appear to be reasonable, when one makes allowance for the different methods of determining the actual blood content. At the end of each experiment, we are, of course, dealing with only a relatively small proportion of the initial dose given remaining in the blood and, although there would appear to be quite a difference between the actual figures observed and calculated, the difference is small, when compared with the total dose injected. The terminal bile content originally observed correlates well with that calculated, using the mathematical model, from the plasma concentrations alone. In all cases, the observed bile content is less than that calculated. This, however, would fit in with the inevitable time delay, due to the biliary tree and cannula dead space.

The terminal liver content of dye is shown in three parts: (i) value for the calculated content of dye, based on the observed plasma concentrations in the mathematical model is given; (ii) the actual amount of bromsulphthalein recovered is given; and (iii) the range for the observed recovery (based on the fact that, with dog liver, only 30 - 40% of the dye present in the liver is actually recovered by the extraction process described in the Methods).

The range for the expected dye content of the liver has been determined in a different manner from that given in the preliminary set of experiments. Two different methods were used in order to determine the range for the expected dye content of the liver. The first took advantage of the fact that Dr. W.H.H. Andrews (at the same time as this particular research project was being carried out) was working independently at St. Mary's Hospital, Paddington, London, on isolated dog liver preparations. In these experiments, the dog liver was being perfused with a bromsulphthalein solution and, at the end of each experiment, Dr. Andrews could determine the amount of dye remaining in his fluid media and the amount recovered in the bile and, by substraction, could give an accurate figure for the amount of dye remaining in the liver. Five liver specimens were transported in a Thermos Flask partially with carbon-dioxide snow on the London to Liverpool Express Passenger Train Service. The trains were met and the specimens collected. Portions of the liver were then put through the extraction process and the dye content determined. The results of these five liver extraction processes are given in Table 7.

Table 7

This table gives the actual amount of bromsulphthalein recovered from dog livers. The first group of experiments were carried out on perfused dog livers obtained from Dr. W.H.H. Andrews at St. Mary's Hospital, London. The expected content of dye is given for these liver specimens and the percentage of dye actually recovered calculated. In the second group, liver slices were incubated in a bromsulphthalein solution and the amount of dye present in and recovered from the liver slices is given, and a percentage recovery calculated. The actual recovery of dye would appear to yield 30 - 40% of the amount present in dog liver.

The recovery of Bromsulphthalein from dog liver.

DOG LIVER RECOVERY

Weight of liver (gms)	Content of Bromsulphthalein (gms)	Total amount of liver examined (gms)	Expected Content (gms)	Amount Recovered	% Recovery
295	47.5	150	24.1	8.5	35.2
275	85.0	160	49.5	15.3	31.3
318	58.0	140	25.5	9.6	37.6
300	70.0	140	32.7	10.6	32.4
340	60.0	180	31.8	11.8	37.1

LIVER SLICE RECOVERY

Amount of Liver Tissue examined	Content of Bromsulphthalein	Bromsulphthalein Recovered	% Recovery
60	19.6	6.0	30.6
40	8.4	3.1	36.7
80	29.7	10.5	35.4
30	4.8	1.8	37.5
100	30.9	12.3	39.8

The range of recovery would therefore appear to yield 30-40% of the actual quantity of dye present.

The second group of experiments consisted of determining the amount of dye present in liver slices which had been incubated in a bromsulphthalein solution of known content. The slices were incubated for a period of a few hours until it appeared as if no further dye was being taken up from the bromsulphthalein solution. This was checked by taking a few drops of the solution and estimating its dye content at half-hourly intervals. The liver slices were then put through the extraction process, in order to determine the amount of dye present. The amount of dye in the slices was taken to be the quantity of dye which had been removed from the bromsulphthalein solution with which the slices had been incubated. The results of five such experiments are given in Table 7. It can be seen from this Table that there is a considerable range for the recovery of bromsulphthalein from dog liver. The recovery rate is less than that which was found in the preliminary experiments using a cat liver. One noticeable feature of the extraction process, using dog liver, was that the liver tissue required more washings with alcohol and ether mixture than cat liver. This may partly be the reason for the smaller amount

of dye recovered in the dog liver series, since more denaturation of protein may have occurred. From this Table, the observed content of dye in the dog liver specimens appears to be 30 - 40% of that expected to be present.

The range for the expected terminal content of liver compared with the calculated amount appears to yield a range which is on the lower side of the calculated amount in the majority of cases. There is, in fact, only one, dog 9, where the lowest content for the expected recovery of bromsulphthalein is higher than that calculated. In dogs 3 and 5, the observed recovery for bromsulphthalein is less than that calculated. In most of the other cases, the mathematically calculated amount tends to approximate nearer to the highest range for recovery, that is, the actual observed amount approximates more closely to 30% than 40% recovery.

Table 6 also details the amount of bromsulphthalein present in the urine. The specimens of urine have been collected at the end of each experiment and therefore this amount of bromsulphthalein is the total amount lost through the kidneys for the whole of the experimental period. It can be seen that in all cases

it is a small amount, usually less than 1% of the dose given, and never more than 1.5%. Also in this Table there is given the results obtained in an abnormal dog. This dog was abnormal in the sense that the plasma disappearance curve did not fall into that anticipated for the normal animal. It was also one in which there was virtually no control period following the acute biliary fistula operation and the injection of bromsulphthalein. During the experiment also, the temperature of the animal rose sharply due to a failure in the heating mechanism of the operating table. There had also been considerable difficulty in cannulating the bile duct in this animal, as well as difficulty in ligating the cystic duct at the time of operation. Altogether, it was felt that the animal had not sufficiently recovered from the operative trauma and, following this, the effect on its heat regulating mechanism. The plasma concentrations calculated and observed in this animal were higher than those of the other 9 dogs. The bile content calculated was 19.7% of the dose given and this is lower than that of even dog 8, in which there also appeared to be less than the usual percentage of dye in the bile after a period of 60 minutes.

In dog 8, the percentage recovery at 60 minutes was 28.2%, whereas in the majority of other animals it exceeded 50%. The observed recovery of dye in dog 10 was also less than expected with a dose of 75 mg. The amount of dye observed in the liver and actually recovered was higher than in any other animal. This yielded a range for recovery which was also higher than the other 9 animals and approximated to the calculated amount. There also appeared to be a greater excretion of bromsulphthalein in the urine although even in this case it was less than 2% of the dose given. The results of dog 10 have not been put through the Electronic Computer, as had the observed plasma concentrations in each of the other 9 animals. The figures given in the Table, have been based on the graphical results alone.

A Simplified Application of the Mathematical Model

In introducing the next two Tables, 8 and 9, it will be of use at this point to explain and simplify the mathematical argument of this work which is contained in the Appendix. The graph of the plasma dye concentration, Figure 32, can be expressed by the equation $C_x = Ae^{-k_1 t} + Be^{-k_2 t}$ where A, B, k_1 and k_2 are constants for the graph and C_x is the plasma

Table 8 (a), (b) and (c)

These tables compare the results in dogs 1, 2 and 3 respectively, using (i) the complex method, i.e. results derived from the mathematical model using the method of least squares and an electronic computer ; and (ii) the simplified method, i.e. results derived from the mathematical model using the graphical method alone.
The blood, liver and bile contents of dye are compared at various times during the period of observation.

(a) Dog 1

Time	Blood Content (mg)		Liver Content (mg)		Bile Content (mg)	
Minutes	Complex	Simplified	Complex	Simplified	Complex	Simplified
0	50.00	50.00	0.00	0.00	0.00	0.00
3	16.92	20.30	32.13	29.10	0.95	0.60
6.25	5.94	8.41	41.08	39.00	2.98	2.59
9	3.05	4.61	42.10	41.10	4.85	4.29
13	1.75	2.60	40.67	40.50	7.58	6.90
16	1.46	2.05	38.99	39.10	9.55	8.85
17	1.38	1.96	38.40	38.60	10.22	9.44
32	1.06	1.40	30.30	30.95	18.64	17.65
42	0.89	1.20	25.87	26.35	23.24	22.45
48	0.81	1.10	23.50	24.23	25.69	24.67
55	0.73	0.99	21.03	21.80	28.24	27.21
60	0.65	0.92	19.42	20.40	29.93	28.68

(b) Dog 2

Time Minutes	Blood Content (mg)		Liver Content (mg)		Bile Content (mg)	
	Complex	Simplified	Complex	Simplified	Complex	Simplified
0	80.0	80.0	0.00	0.00	0.00	0.00
3	31.45	31.60	47.23	46.40	1.32	2.00
6	13.45	12.90	62.77	62.90	3.98	4.20
9	6.37	5.31	66.57	67.10	7.06	7.59
12	3.71	3.64	66.02	67.40	10.27	10.34
15	2.66	2.49	64.01	66.40	13.33	11.11
20	2.06	1.98	59.70	62.30	18.24	15.72
30	1.70	1.66	51.31	53.80	26.99	24.54
40	1.42	1.42	44.06	46.40	34.52	32.17
50	1.24	1.24	37.78	40.00	40.98	38.76
60	1.05	1.05	32.42	34.40	46.53	44.54

(c) Dog 3

Time Minutes	Blood Content (mg)		Liver Content (mg)		Bile Content (mg)	
	Complex	Simplified	Complex	Simplified	Complex	Simplified
0	100.00	100.00	0.00	0.00	0.00	0.00
3	27.46	30.20	70.67	68.05	1.87	1.75
6	8.73	10.38	85.94	85.90	5.33	3.72
9	3.84	4.62	87.08	87.00	9.08	8.38
15	2.12	2.42	81.53	82.50	16.35	15.08
20	1.88	2.10	76.11	77.60	22.01	20.30
30	1.63	1.81	66.18	68.00	32.19	30.19
40	1.41	1.60	57.53	59.70	41.06	38.70
45	1.32	1.51	53.64	56.00	45.04	42.49
61	1.05	1.22	42.88	45.50	56.07	53.28
75	0.87	1.02	35.25	37.90	63.88	61.08
90	0.70	0.84	28.57	31.20	70.73	67.96
100	0.61	0.74	24.84	26.40	74.55	72.86
105	0.57	0.69	23.16	25.65	76.27	73.66
120	0.46	0.57	18.77	21.15	80.77	78.28

concentration at time t, and e is the natural base. The form of this graph is satisfied by saying that the liver will accept bromsulphthalein at a proportionality rate which we may call 'a'; it will return dye to the plasma at a proportionality rate 'b'; and that it will excrete dye into the bile at a proportionality rate 'h'. By "proportionality rate" is meant the rate of transfer of dye per unit load in the appropriate direction. Thus, we are able, knowing these rates, to calculate how much dye has passed to the liver and thence to the bile. It can be shown (Cf. Appendix for proofs) that 'a' is equal to $\frac{Ak_1 + Bk_2}{A + B}$ and 'h' is equal to $\frac{K_1 K_2}{a}$ and $b = k_1 + k_2 - (a - h)$.

The dye contents of plasma, liver and bile at any time depend upon the values of these three proportionality rates which are constant for any particular experiment. They are calculated from the parameters (A, B, k_1 and k_2) of the graph of plasma concentration only. These parameters may be determined graphically by simple measurement or by more complex mathematical procedures from the graph of the "best fit". Table 8, (a), (b) and (c) show

Table 9

The values for the mathematical constants k_1, k_2, a, b and h, derived using the method of 'least squares' and an electronic computer for the nine acute biliary fistula dogs, 1 - 9.

The dose of bromsulphthalein is also given.

The constants of an abnormal dog (10) have been inserted for comparison, but these constants have only been derived graphically.

Mathematical Constants

Dog	Slope of Initial Phase k_1	Slope of Second Slower Phase k_2	Blood to Liver a	Transfer of Bromsulphthalein Liver to Blood b	Liver to Bile h	Dose of Bromsulphthalein (mg)
1	0.384	0.0159	0.371	0.0125	0.0164	50
2	0.328	0.0153	0.317	0.0105	0.0158	80
3	0.453	0.0140	0.442	0.0107	0.0143	100
4	0.359	0.0104	0.342	0.0165	0.0109	50
5	0.457	0.0211	0.427	0.0285	0.0226	42.5
6	0.297	0.0197	0.290	0.0065	0.0202	90
7	0.325	0.0150	0.306	0.0181	0.0159	70
8	0.313	0.0066	0.299	0.0140	0.0069	35
9	0.408	0.0145	0.387	0.0202	0.0153	50
10	0.198	0.0046	0.187	0.107	0.0049	75

comparisons between the values given by the two methods for the dogs 1, 2 and 3, in Table 6. It takes a skilled numerical analyst over a day to provide the data for an Electronic Computer, whereas the simple method may be completed within an hour. It can be seen that in these three animals the results are similar and it is possible that the simpler method might well be adequate for clinical purposes. The proportionality constants which have been derived using the Electronic Computer in the 9 normal animals with acute biliary fistula following a 5 mg./Kg. dose of body weight is given in Table 9. There appears to be a reasonable range of these constants in the 9 animals with the exception of dog 8, where the slope of the second slower phase, k_2 is much less than any of the other normal animals. This is also reflected by the much lower rate of transfer of bromsulphthalein from the liver to the bile, compared with all the other animals. The terminal calculated contents of dye reflect this difference in the proportionality rates as shown in Table 6. Dog 10 has had the constants calculated by a graphical method alone but in this animal which was considered to be abnormal during the period of

observation, there is a much slower slope of the initial phase and a slope of the second slower phase which is of the same order as dog 8. It is, however, to be noted, that in this particular animal the rates of transfer of bromsulphthalein from the blood to the liver is less, and this is again reflected in the terminal blood contents shown in Table 6, although it is not strictly correct to compare these figures, since the dose of bromsulphthalein given, although 5 mg./Kg. body weight, is different.

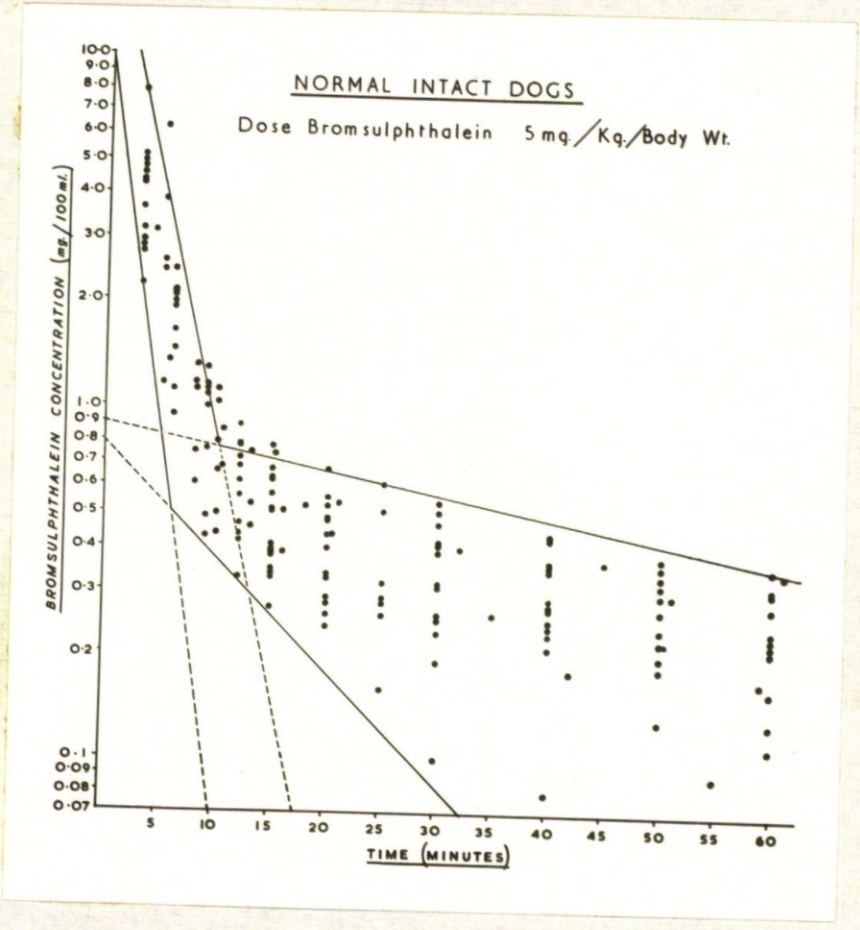
The Normal Plasma Disappearance Curve in Dogs

Figure 39 shows the logarithm plasma concentration of dye in relationship to time in 18 normal dogs. In these experiments, the animal was anaesthetised in the usual manner with Themalon and Nembutal and then a small incision was made over one or other femoral veins. A 5 mg./Kg. dose of bromsulphthalein was given intravenously into another vein, usually on the forelimb. The plasma concentrations were then determined at various time intervals. It can be seen that the observed concentrations tend to follow a similar pattern when plotted against time. The pattern is so uniform that an outside limit of normal which would show an initial rapid phase and a

Figure 39

A scattergram of the plasma concentrations of bromsulphthalein in relation to time in 18 normal intact dogs.

The outside limit for these results are given in the form of a double exponential graph, similar to that depicted in Figure 32.


NORMAL INTACT DOGS
Dose Bromsulphthalein 5 mg./Kg./Body Wt.
BROMSULPHTHALEIN CONCENTRATION (mg./100 ml.)
100
9·0
8·0
7·0
6·0
5·0
4·0
3·0
2·0
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0·09
0·08
0·07
5
10
15
20
25
30
35
40
45
50
55
60
TIME (MINUTES)

second slower phase can be drawn. There are a series of points between these two lines which would fall on the "bend". The lines which represent the outside limits of normal in these animals have been used in subsequent sections to show what is considered to be the upper limit of the normal intact dog. In the majority of subsequent experiments, the animals have been used on several occasions, for estimations of the serial plasma concentrations of bromsulphthalein, sometimes as a control curve and on other occasions when other substances have been given before, at the same time, or following bromsulphthalein. In a few cases, however, there was no control curve available and hence the usefulness of these lines obtained from the "scattergram" in Figure 39, to give an idea of the expected normal limit.

From the point of view of the mathematical model, it is reassuring to know that the disappearance curves in the normal intact animal appear to follow a similar pattern to that observed in the acute biliary fistula animal.

Continuous Infusion Experiments

Continuous infusions of bromsulphthalein have been used for determining the estimated hepatic blood flow. Following a continuous infusion of bromsulphthalein, it takes a long time before a steady plasma concentration is actually obtained and some of the errors in using this technique for determination of liver blood flow may be due to the fact that a steady plasma concentration has not been obtained. Although it is not the purpose of this series of experiments to determine liver blood flow, a cardiac catheter was used in order to obtain samples of the hepatic venous blood, so in three animals an estimate of the liver blood flow was carried out and the results of these experiments are given in Table 10. The values obtained in these three animals correspond satisfactorily with the range given previously in Table 1, for the estimated hepatic blood flow using the bromsulphthalein technique.

Figure 40 demonstrates the importance of the continuous infusion. In the case of 578, a continuous infusion of 0.63 mg. per minute was given and it can be seen that even after three hours there was still

Table 10.

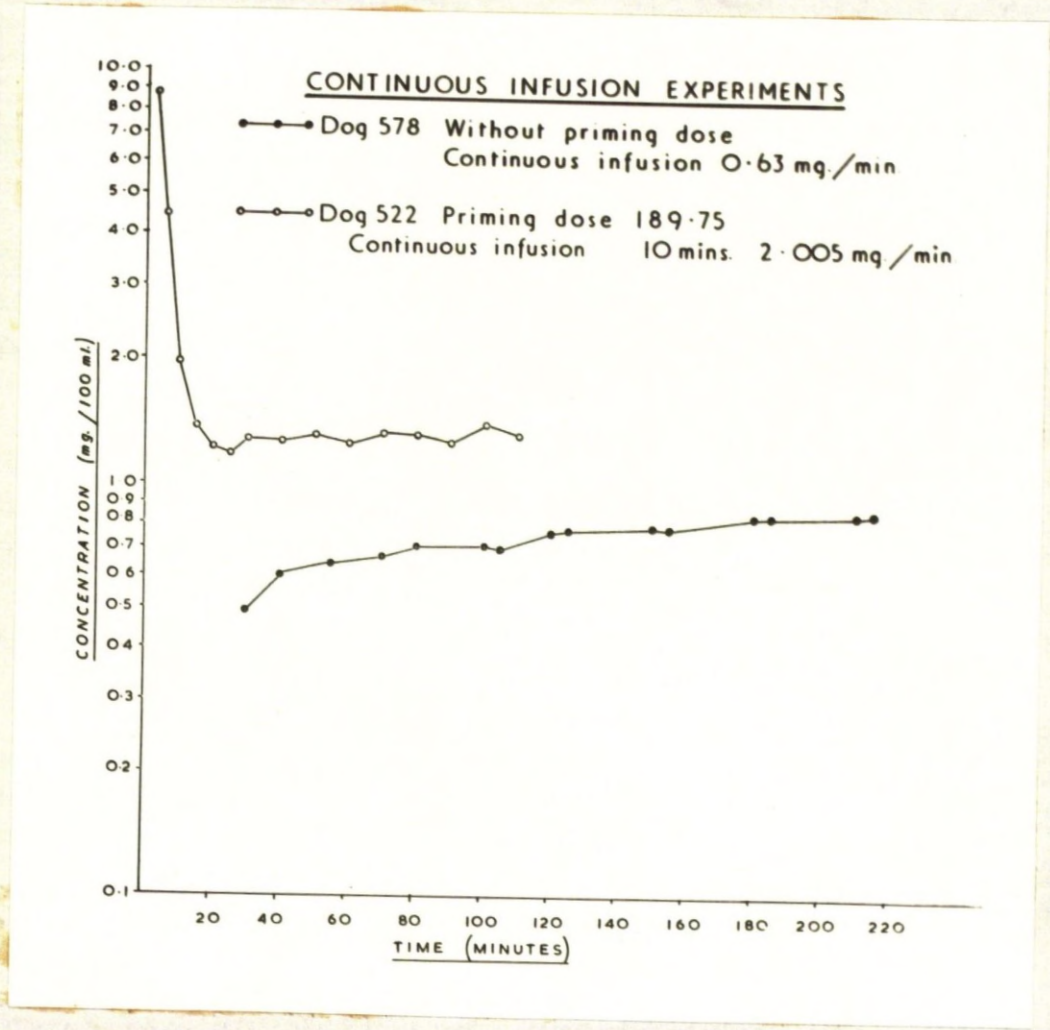
The estimated hepatic blood flow in three dogs. The results would appear to correspond well with those given in Table 1, where the bromsulphthalein technique for the estimation of the hepatic blood flow has been used.

Estimated Hepatic Blood Flow

Weight of Dog Kg.	Assumed plasma volume (50 ml/Kg)	Number of Observations of A-V Differences	Infusion Rate mg/mins.	Average A-V Difference	E.H.B.F. ml/mins.	E.H.B.F. ml/Kg/mins
14	700	6	0.964	0.153	630	45
8	400	6	0.872	0.287	304	38
12	600	8	1.645	0.318	516	43

Figure 40

This figure shows the results of two continuous infusion experiments. In the case of dog 578, a continuous infusion of 0.63 mg./min. was given without any priming dose. It can be seen that a steady plasma concentration has not been obtained even at 200 minutes. It is also to be noted that if two readings are taken closely together then they may well give the impression of a steady level after 100 minutes. The second experiment, dog 552, was given a priming dose of 189.75 mg. of bromsulphthalein and a continuous infusion 10 minutes after the priming dose, and the rate was 2.005 mg./min. Plasma levels appear to approximate to a steadier plateau much more quickly than the continuous infusion alone (e.g. dog 578).


CONTINUOUS INFUSION EXPERIMENTS
Dog 578 Without priming dose
Continuous infusion 0·63 mg./min
Dog 522 Priming dose 189·75
Continuous infusion 10 mins. 2·005 mg./min
CONCENTRATION (mg./100 ml.)
10·0
9·0
8·0
7·0
6·0
5·0
4·0
3·0
2·0
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
20
40
60
80
100
120
140
160
180
200
220
TIME (MINUTES)

not a steady plasma level. In fact, in this particular experiment, over four hours elapsed before a steady plasma concentration was obtained. Another interesting point to be noted is that readings taken closely together may give a value which indicates that the actual plasma level has been reached. For instance, the last five group of points in this Figure for dog 578, indicate plasma concentrations of approximately the same value, but over the time period it can be seen that the plasma concentration is, in fact, steadily rising. Normally, a small priming dose is given, the dose often being 50, 100 or 150 mg. of bromsulphthalein. This may indeed shorten the period of time that elapses before a steady plasma concentration is reached, but even so, it is doubtful if it can be obtained before approximately $2\frac{1}{2}$ - 3 hours after the commencement of the infusion, when given as a random amount. The mathematical model enables one to predict a satisfactory priming dose for any fixed rate of continuous infusion, in order that a steady plasma level may be obtained more rapidly. Although the example given in Figure 40 (dog 522) does not exactly give a steady plasma level, it does indicate how closely this can be approximated in a much shorter time. In this

particular experiment, a priming dose of 189.75 mg. of bromsulphthalein was given at zero time and a continuous infusion of 2.005 mg. commenced at ten minutes. Unfortunately, these quantities were not the values requested by Mr. A. Young (Department of Applied Mathematics) who suggested that a priming dose of 189.78 be given and a continuous infusion at 10 minutes of 2.0 mg. Table 11 gives the priming dose for dog 522 and the time of commencement of the continuous infusion.

Giving the exact priming dose requested proved difficult on this occasion, due to the high concentration of bromsulphthalein in the ampoule supplied (50 mg. per ml.) By suitable dilution of the solution however, without greatly increasing the volume of fluid injected, this error could be avoided. Even with a good continuous infusion machine it is sometimes difficult to give an exact quantity and in the experiment carried out, instead of an amount of 2.0 mg. per minute being given, 2.005 mg. per minute was in fact given. However, it can be seen that the readings do tend to give a straight line, i.e. almost a steady plasma concentration at about one hour. The fact that the mathematical model can be adapted to be able to give the necessary information to obtain a steady plasma level after a

Table 11

A calculated continuous infusion experiment. In the case of dog 522, the calculated primary dose using the mathematical model is given at zero time and the times of commencement of a continuous infusion of 2.0 mg./min. of bromsulphthalein, with the different priming doses, are given in this Table.

Calculated continuous infusion Dog 522.

Priming dose of Bromsulphthalein (mgs.)	Time of Commencement of Continuous Infusion of 2.0mg/min.
185.32	8 mins.
189.78	10 mins.
196.67	13 mins.
201.22	15 mins.
216.23	21 mins.

short interval of time (from information derived from a previous single injection control plasma distribution curve) may well make it useful as a preliminary to determining the estimated hepatic blood flow using bromsulphthalein.

Discussion.

Bromsulphthalein normally combines with the albumin fraction of the plasma (Brauer and Pessotti, 1948, 1949; Ingelfinger, Bradley, Mendeloff and Kramer, 1948). In a reference to some unpublished observations (Cohen, Althausen and Giansiracusa, 1956) this finding has been confirmed by paper electrophoresis of normal sera containing S^{35} tagged bromsulphthalein. The dye also forms stable complexes, with various soluble as well as granular protein fractions from the liver (Brauer and Pessotti, 1950). It has been shown by Shore and Zilversmit, 1954, that blockade of the reticulo-endothelial system has no effect on bromsulphthalein clearance. Bromsulphthalein had been localised in the hepatic polygonal cells of rat liver by Krebs and Brauer, 1949 and Mendeloff, 1949, who found the fluorescence of Rose Bengal only in the hepatic polygonal cells and

inferred that bromsulphthalein would be treated in a similar fashion. Cantarow, Wirts, Snape and Miller (1948) observed that the process of removal of bromsulphthalein from the circulation involves uptake and storage by the liver, followed by transfer into the biliary tract; they showed that the excretion of bromsulphthalein into the bile, lags behind its disappearance from the blood stream, and surmised that this delay represents storage of the dye. Other workers, Brauer and Pessotti (1950) have also noted a lag in biliary excretion of bromsulphthalein, indicating its storage in the body. These facts suggest that the process of biliary excretion of bromsulphthalein involves its transfer from combination with albumin in the blood to a combination with the proteins of the hepatic polygonal cells. This is followed by transfer into the biliary tract.

When the recovery of S^{35} - tagged with bromsulphthalein in plasma abd bile by colorimetric and radio-active methods was compared, it was shown that, although the recovery of dye from plasma was identical, this was not the case with bile (Brauer and Pessotti, and Krebs, 1955). It appeared that only two-thirds of the dye present was detected colorimetrically as

compared with that by radio-active methods. Subsequent work by these workers has shown that the bromsulphthalein is excreted into the bile as various breakdown products of bromsulphthalein, known as I, II and III. In the series of experiments detailed in this Section, it would appear that the colorimetric method derived (see METHODS) yields a higher recovery of dye than that used by Brauer and his colleagues (see Table 6). In the experiments using S^{35} - tagged bromsulphthalein, less than 2% of the S^{35} was detected as inorganic sulphate in the bile. Since a continuous infusion of bromsulphthalein was used in these experiments, the loss as inorganic sulphate following a single injection is probably less. It would also appear that during its passage through the liver a proportion of the bromsulphthalein is altered so that it can no longer be detected colorimetrically.

It has been shown by Lorber, Oppenheimer, Shay and Lynch and Siplet (1953) that in normal anaesthetised dogs following the 5 mg./Kg. body weight dose of bromsulphthalein, the entero-hepatic circulation of the dye is negligible, even when the dose was put into the duodenum. The experiment carried out on dog 602, illustrated in Figure 30 confirms this finding.

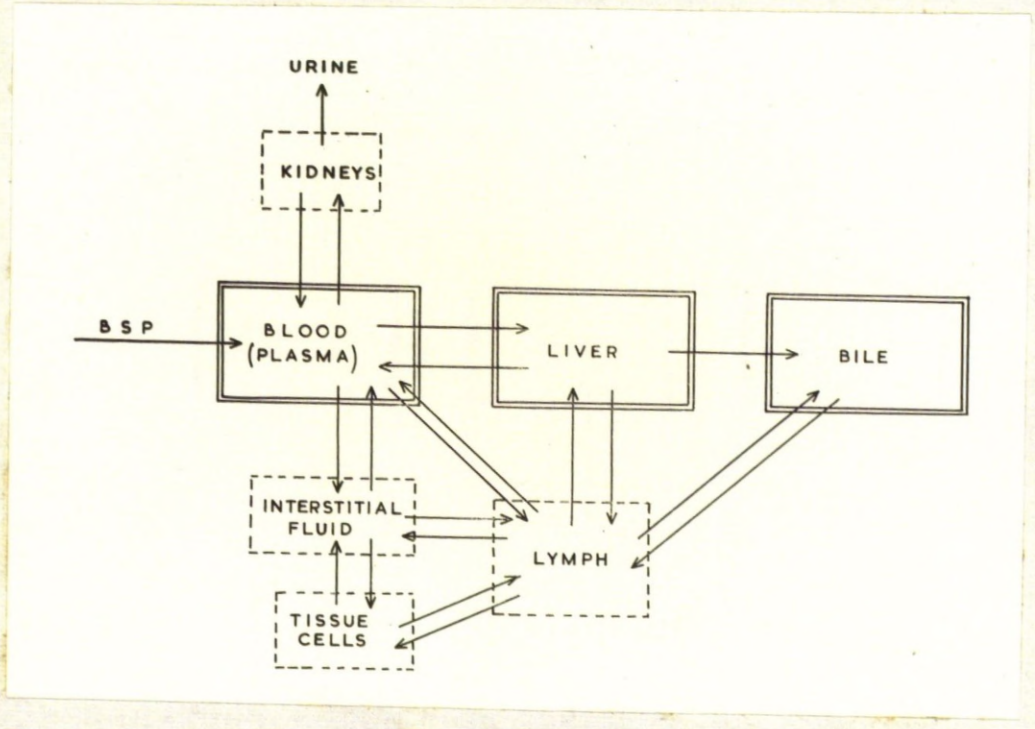
In the series of experiments described in the nine, normal, acute biliary fistula animals, there have only been small quantities of dye present in the urine. Never more than 1 mg. and very rarely more than 1% of the dose given, the average being 0.6%. In human subjects, according to Norcross, White and Bradley (1951), the urinary excretion of bromsulphthalein ranged between 0.8 and 1.9% of the dose given (5 mg./Kg.), the average being 1.1%. With larger doses and continuous infusions of bromsulphthalein, this loss is greater but under the conditions of the present experiments can be considered negligible. In hepatectomised, nephrectomised and eviscerated dogs, it was found by Cohn, Levine and Streicher (1947) and Conn, Levine and Kolinsky (1948) that there was a considerable loss of bromsulphthalein to the extra hepatic tissues. The doses given to these animals were greatly in excess of a single standard 5 mg./Kg. dose, and from their results it can be shown that the rate of transfer to the tissues per unit plasma is still very low.

It seems reasonable therefore to see bromsulphthalein as being distributed between the three compartments of blood, liver and bile in the conditions of the

Figure 41

The possible sites of distribution of bromsulphthalein.

Some of these are known and some are possibilities. The principal sites are indicated by solid lines and the other sites by dotted lines. The mathematical model only takes into account the overall transfer between blood, liver and bile.


URINE
KIDNEYS
BSP
BLOOD
(PLASMA)
LIVER
BILE
INTERSTITIAL
FLUID
LYMPH
TISSUE
CELLS

present experiments (Figure 41). This view receives support from experiments performed by Brauer and Krebs and Pessotti (1950). Using continuous infusions of S^{35} tagged bromsulphthalein, these workers were able to obtain nearly quantitative recovery from the liver, blood and bile and in continuous infusion experiments, extra hepatic loss of dye is maximal. Even with large continuous infusions of S^{35} tagged bromsulphthalein, 80% of the total dye could be accounted for in the terms of hepatic extraction (Brauer, Pessotti and Krebs, 1955). A large part of the extra hepatic loss was detected in the skeletal muscle. There are, however, no reports of any such loss, following a single injection of bromsulphthalein, 5 mg./Kg. body weight. The agreement between the observed content of bile and the content calculated mathematically is good (Figure 38 and Table 6), especially as 100% colorimetric recovery is impossible. The presumptive liver content based upon observed blood and bile recovery is in good agreement with the calculated content and the terminal liver content (on the basis of a 30 - 40% recovery) is again in fair agreement with the calculated content.

A mathematical relationship between the quantity

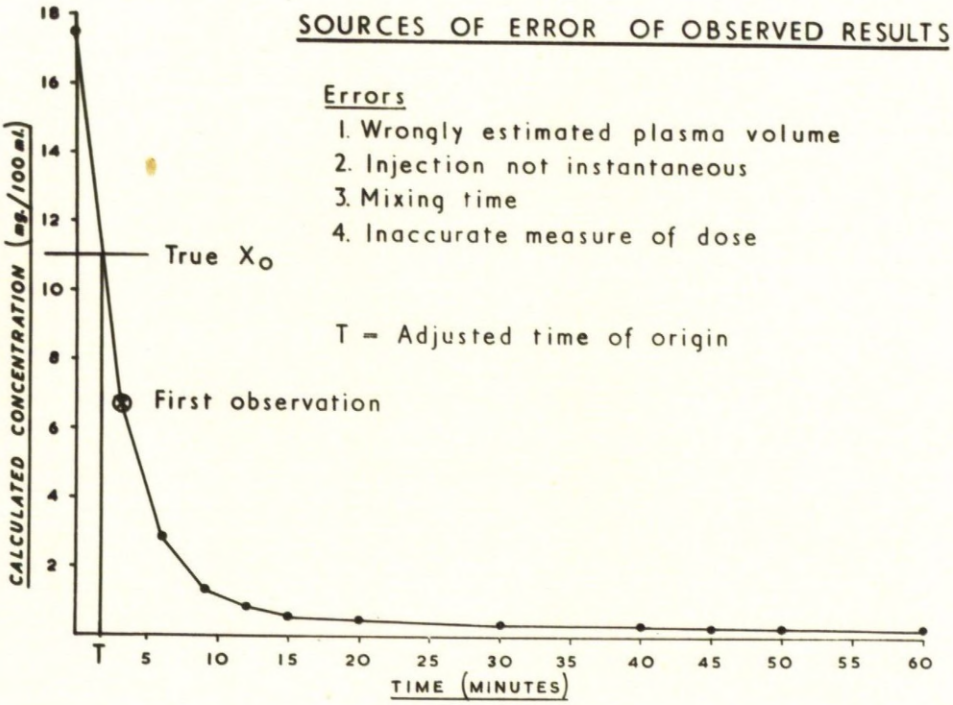
of bromsulphthalein injected into the blood, the amount taken up by the liver, and the amount of the dye excreted in the bile, is given in the Appendix. In order to obtain these quantities with mathematical precision in any experiment, either a time-consuming analytical procedure is required, or use has to be made of the electronic computer (an expensive process). The simplified method described in this Section would appear to give a reasonable approximation (sufficient for a clinical test) to the complex, mathematically determined temporal distribution of bromsulphthalein (Table 8 a, b & c). It would seem that in this way one may describe quantitatively the "ability" of the liver cell to deal with the exogenous dye bromsulphthalein, both in uptake and excretion. It is suggested that the application of the modified bromsulphthalein test will yield more information regarding the ability of the liver to excrete this exogenous dye than the estimation of the 45 minute retention of bromsulphthalein (a standard clinical test).

The possible sites of distribution of bromsulphthalein have been given in Figure 41 and some of the errors which occur in the observed results

Figure 42

The graph details some of the errors which occur following the intravenous administration of bromsulphthalein and the subsequent estimation of the observed concentrations of dye at various times following the injection.

SOURCES OF ERROR OF OBSERVED RESULTS



Errors

1. Wrongly estimated plasma volume
2. Injection not instantaneous
3. Mixing time
4. Inaccurate measure of dose

T = Adjusted time of origin

as they might affect of have to be taken into account by the mathematical model, have been depicted in Figure 42. It is also of interest to note that an almost steady plasma concentration of bromsulphthalein has been obtained using an initial dose and a continuous infusion of fixed amounts based on the mathematical assumption that the bromsulphthalein is distributed in the plasma, in the blood, liver or bile. If there was any great loss to any extra hepatic source, then surely the steady plasma level predicted could not have been obtained.

At the time this work was being carried out, the electronic computer used was housed at Manchester University and because of this the results obtained in the subsequent Sections unfortunately could not be analysed by the computer. The facilities for analysing these results would now be available by making use of the electronic computer in this University, but they have, so far, not been programmed for use in a computer. A more detailed appraisal of the value of the mathematical model will be given in the General Discussion at the end of the Results.

Mathematical Treatment of Results

Mathematical treatment of results. Suppose that at time t, x, y and z be the total amounts of BSP in the blood, in the liver and in the bile. It is assumed that under normal conditions :-

(i) all rates of transfer of BSP are proportional to the amounts of BSP from which the transfer occurs;

(ii) the amount of BSP transferred to extra-hepatic or extra-vascular tissue is negligible.

Let the constants of proportionality for the rates of transfer (i) from blood to liver, (ii) from liver back to blood, and (iii) from liver to bile be a, b and h respectively. Then following the completion of an injection of BSP into the blood, the above transfers are governed by the equations.

$$\left.\begin{aligned} \frac{dx}{dt} &= -ax + by \\ \frac{dy}{dt} &= -(b + h)y \\ \frac{dz}{dt} &= hy \end{aligned}\right\} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (1)$$

These equations have the solutions

$$x = \frac{(a - k_2)\,x_o - by_o}{k_1 - k_2}\, e - k_1 t + \frac{(k_1 - a)\,x_o + by_o}{k_1 - k_2}$$

$$y = \frac{(k_1 - a)\,y_o - ax_o}{k_1 - k_2}\, e - k_1 t + \frac{(a - k_2)\,y_o + ax_o}{k_1 - k_2} \quad ..(2)$$

$$z = x_o + y_o + z_o - y - y$$

The general solution of x in equation (2) is

$$x = Ae^{m_1 t} + Be^{m_2 t}$$

where m_1 and m_2 are roots of

$$k^2 + (a + b + h)k + ah = 0$$

but as the values m_1, m_2 are always negative in physiological conditions, it is more convenient to write

$$x = Ae^{-k_1 t} + Be^{-k_2 t}$$

where $k_1 = m_1$ and $k_2 = m_2$

and therefore k_1 and k_2 are roots of the equation

$$k^2 - (a + b + h)k + ah = 0 \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots(3)$$

It is found experimentally that k_1 is much greater than k_2 and so $e - k_1 t$ attenuates very much more quickly than $e - k_2 t$ and the last

term in the equation for x, in (2) above, is ultimately dominant. For large values of t, the first term may be neglected, so that

$$\ln x \doteqdot \ln \left(\frac{((k_1 - a)\, x_o + by_o)}{k_1 \quad k_2} \right) - k_2 t \qquad \ldots\ldots\ldots\ldots\ldots\ldots (4)$$

Provided that the ratio

$$R = \frac{(a - k_2)\, x_o - by_o}{(k_1 - a)\, x_o + by_o}$$

is sufficiently large, the first term in x, (2 above) will be dominant initially, and thus for small values of t

$$\ln x \doteqdot \ln \left(\frac{((a - k_2)x_o - by_o)}{k_1 - k_2} \right) \qquad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (5)$$

Equations (4) and (5) indicate that the graph of ln x shows an asymptotic approach to the line given by (4) for large values of t, an asymptotic approach to the line (5) for small values of t, and an intermediate part where the graph changes from one asymptote to the other. This gives a graphical method of determining k_1 and k_2 (see Figure 32); and, if x_o and y_o be known, a, b and h can be deduced.

As the ratio R decreases, however, the asymptote (5) is less and less likely to be evident and since R decreases as y_o increases, it follows in practice that if a second or third

injection is given before all the BSP from previous injections is excreted from the liver, the curve of ln x subsequent to later injections will show the values of k_1 less accurately. This explains why graphically (Figure 62) there appears to be a greater alteration of the initial rapid phase following the second and third injections than of the later slower phase, which is apparently unaltered. The constants of proportionality for the rates of transfer (i) from blood to liver, (ii) from liver to blood and (iii) from liver to bile, namely a, b and h respectively, are unaltered. Following the first injection the rate of the rapid phase is so high that there are seldom more than two points on the graph which contain large contributions from the first term in x (equation 2 above); after subsequent injections, these early points contain relatively larger contributions from the second term in x, so that the ratio R is not large. When the graphs obtained following the second and third injections are examined (Figure 62) it can be seen that the curves tend to identify the line joining the first few points on it as the asymptote given by equation 5 above, whereas in reality after second and later injections these points lie on the curves portions (BC) of the graph joining the two asymptotes (AB) and (CD).

In practice, an injection takes a few seconds to administer and before it is completed some BSP will have found its way out of the blood. Strictly speaking, therefore, y_o and z_o as defined above are not zero but if for the present purpose it be assumed that they are, then x_o is equal to the total amount of BSP injected. This is equivalent to assuming that the injection is made instantaneously. With this limitation, then

$$\left.\begin{aligned} x &= x_o \left(\frac{a - k_2}{k_1 - k_2} e^{-k_1 t} + \frac{k_1 - a}{k_1 - k_2} e^{-k_2 t} \right) \\ y &= \frac{a x_o}{k_1 - k_2} (e^{-k_2 t} - e^{-k_1 t}) \\ z &= x_o - x - y \end{aligned}\right\} \quad \ldots\ldots(6)$$

In both (2) and (6) the third equation involves the assumption that all the BSP is to be found in the blood, liver or bile, the extra-hepatic or extra-vascular uptake being regarded as negligible.

In the preceding part of the mathematical treatment, the total amounts of BSP in the blood, liver and bile have been considered. Experimentally, the concentrations of BSP at various times (t) are obtained. From these plasma concentrations, it is possible to derive the plasma content of dye as explained in the following paragraph.

Assuming the plasma volume remains constant throughout the experiments, the observed plasma concentrations of BSP (C_x) say, are directly proportional to the total amount of x in the circulation. Let the factor of proportionality be λ, so that

$$C_x = \lambda x \quad \ldots\ldots\ldots\

(iii) The relations $k_1k_2 = ah$ and $k_1 + k_2 = a + b + h$, which follow from (3) next yield h and b.

(iv) Finally, since from (9)

$A + B = \lambda x_o$

and x_o (the dose of BSP) is known, λ can be determined.

Thereafter the experimentally- found concentrations (C_x) can be converted into estimates of actual quantities (x); finally, y and x (liver and bile content of BSP respectively) can be calculated from the second and third equations of (6).

B. The Effect of Bromsulphthalein and Other Substances (Benemid, Bilirubin, Dehydrocholin and Biligrafin) on Bile Volume.

The Choleretic Effect of Bromsulphthalein

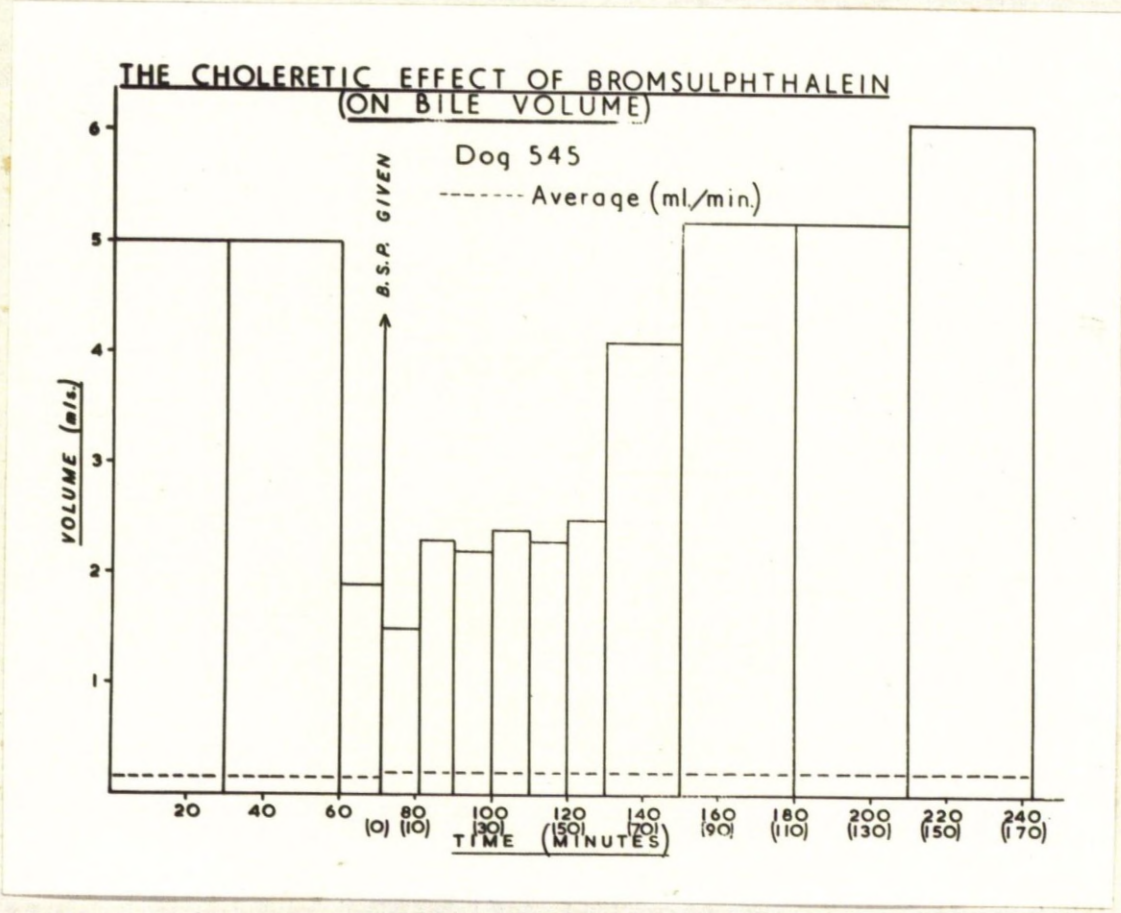
As has been seen in the case of the acute biliary fistula, cats 8 and 9, following an injection of bromsulphthalein, there is a rapid increase in the amount of bromsulphthalein excreted in the bile to reach a maximum at some time between 30 and 45 minutes, after which it falls to a lower level. In these cats, it was noted that the bile volume was increased during the time following a bromsulphthalein injection as compared with the control bile volume. The result in these two experiments has been given in Table 2, where it is shown that in the early part of the experiment (the first 60 minutes after bromsulphthalein has been injected), there is a percentage increase in bile volume of 25.9% in one case and 40% in the other. If the total volume increase for the duration of these experiments is 110 and 120 minutes respectively then the rate of overall increase is approximately 10% in one case and 20% in the other. Although the bile had been collected for estimation of bromsulphthalein by various workers, there does not appear to be a specific reference to the increase in bile volume until Sperber (1957) observed a choleresis in

Figure 43

The Choleretic Effect of Bromsulphthalein (Dog 545).

The volume of bile was noted for a control period of 71 minutes. Bile samples were then collected at approximately ten minute intervals for sixty minutes, afterwards being collected at longer intervals (15 - 33 minutes). An arrow indicates the time at which bromsulphthalein (5 mg./Kg.) was given.

At the bottom of the graph, the average volume rate (mls./min.) during the control period and during the whole period following bromsulphthalein are respectively indicated by a dotted line.


THE CHOLERETIC EFFECT OF BROMSULPHTHALEIN
(ON BILE VOLUME)
Dog 545
------- Average (ml./min.)
B.S.P. GIVEN
VOLUME (mls.)
1
2
3
4
5
6
20
40
60
80 (10)
100 (30)
120 (50)
140 (70)
160 (90)
180 (110)
200 (130)
220 (150)
240 (170)
(0)
TIME (MINUTES)

chickens after the administration of bromsulphthalein.

The effect of a 5 mg./Kg. dose of bromsulphthalein in dog 545 is shown in Figure 43, in which the volume of bile in millilitres is given over the time periods during which bile was collected. There was a control period of 71 minutes in this case and during the first 60 minutes a volume of 5 mls. of bile for each 30 minute sample was obtained and during the last 11 minutes, 1.9 mls. of bile was obtained. Following the intravenous injection of bromsulphthalein, bile was collected at approximately 10 minute intervals for a period of 60 minutes, after which it was collected at 20 or 30 minute intervals. There is an increased volume of bile in each of the 10 minute periods and also during the subsequent periods of observation. The dotted lines given in the lower part of the Table during the control period give the average volume during the 71 minute control period, i.e. 0.168 mls./min., and the dotted line following the injection of bromsulphthalein gives the average volume rate of 0.203 mls. per minute, but on this scale the difference is not particularly marked. When the bile rate in mls. per minute during the time periods of observation are given, as

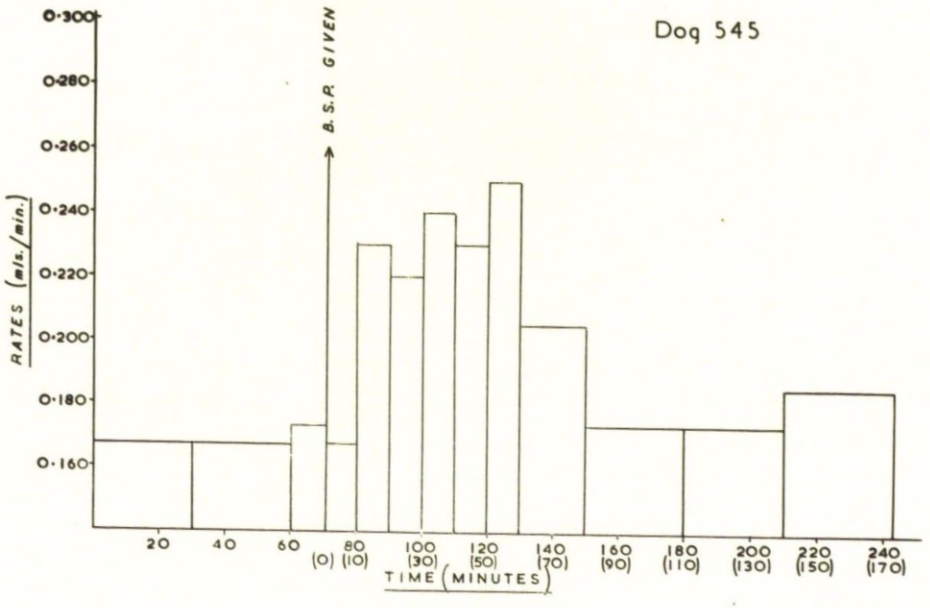
Figure 44

The Choleretic Effect of Bromsulphthalein

The bile volume rate in millilitres per minute is compared in a similar manner to Figure 43.

The control volume rate is given, followed by the volume rate after the injection of bromsulphthalein (indicated by an arrow). It can be seen that after a short time interval delay there is a marked increase in the rate of bile flow.

THE CHOLERETIC EFFECT OF BROMSULPHTHALEIN (ON BILE VOLUME)



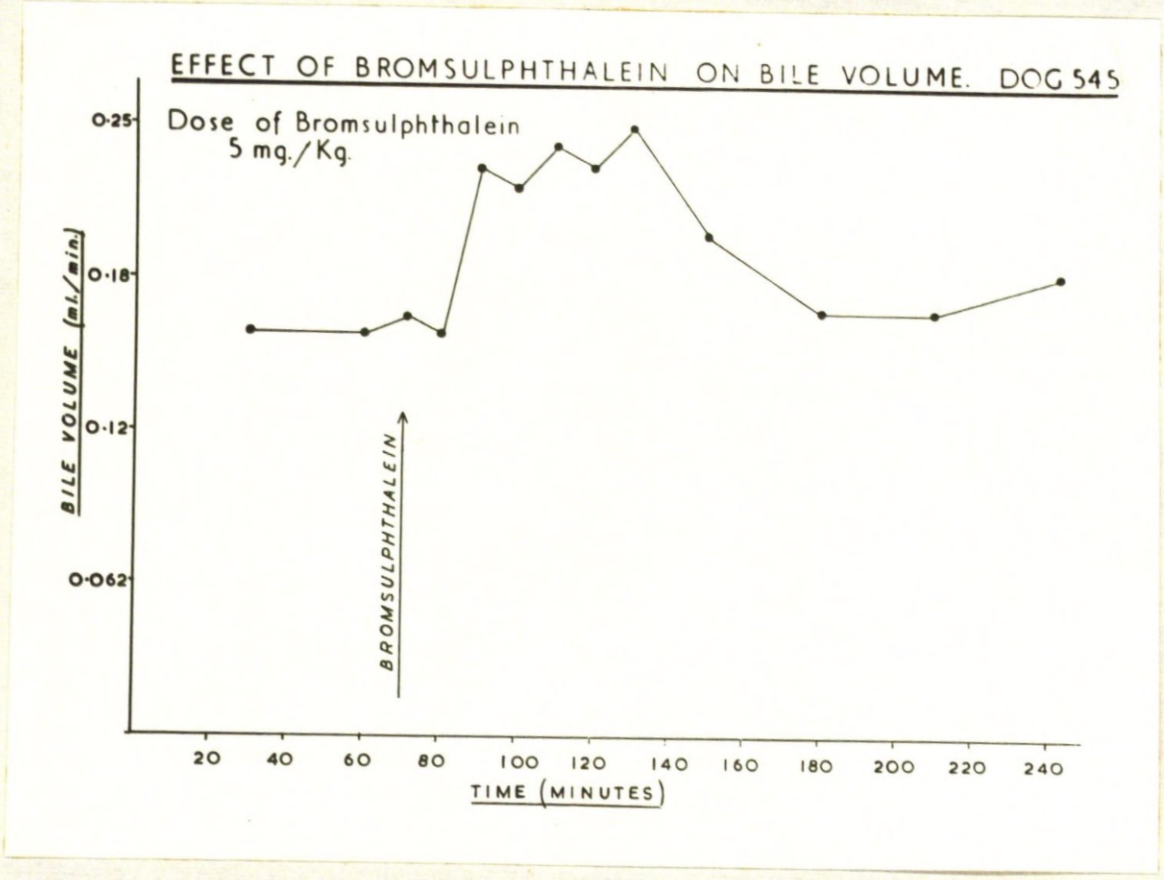
shown in Figure 44, it can be seen that during the period just prior to bromsulphthalein, there was a slight increase in the average rate as compared with that which occurred during the previous 60 minutes. The first 10 minute specimen of bile after the injection of bromsulphthalein gives a value which corresponds to the 60 minute period of control observation. Following this, the bile volume is markedly increased and the rate reached a maximum peak, somewhere between 50 - 60 minutes after the injection of bromsulphthalein. The bile volume is still increased, compared with the control period for a total of 173 minutes after the injection of bromsulphthalein had been given. Due to the inevitable dead space in the bile ducts and cannula there is a short delay before the choleretic action of bromsulphthalein is noted. This is followed by a maximum effect and then a gradual falling of the rate.

Figure 45 shows the bile volume rate in millilitres per minute in a graphical form. Prior to the bromsulphthalein being given, the control volume is reasonably steady, with the exception of a slight increase in the control rate just before the

Figure 45

The Effect of Bromsulphthalein on Bile Volume.

The bile volume rate in millilitres per minute is compared in dog 545 in the form of a graph. Here, the effect of bromsulphthalein is more striking. It can be seen that there is an initial marked effect after a slight time delay followed by a gradual fall. Towards the end of the experiment there is a slight rise in the bile volume rate which may possibly by due to the entero-hepatic circulation of bromsulphthalein.


EFFECT OF BROMSULPHTHALEIN ON BILE VOLUME. DOG 545
Dose of Bromsulphthalein
5 mg./Kg.
BILE VOLUME (ml./min.)
0·25
0·18
0·12
0·062
BROMSULPHTHALEIN
20
40
60
80
100
120
140
160
180
200
220
240
TIME (MINUTES)

injection of bromsulphthalein. The first 10 minute specimen after the injection of bromsulphthalein is equal to the control volume rate. This is followed by a rapid rise in the rate to reach a maximum 60 minutes after the bromsulphthalein has been given. There is then a gradual fall in the choleretic effect of bromsulphthalein but even 140 minutes after the bromsulphthalein has been given, the effect is still apparent, since the bile volume rate is greater than the control volume. It is interesting to note that the bile volume increases again slightly between 140 to 173 minutes and it raises the possibility of the entero-hepatic circulation of bromsulphthalein playing a part in this slight increase.

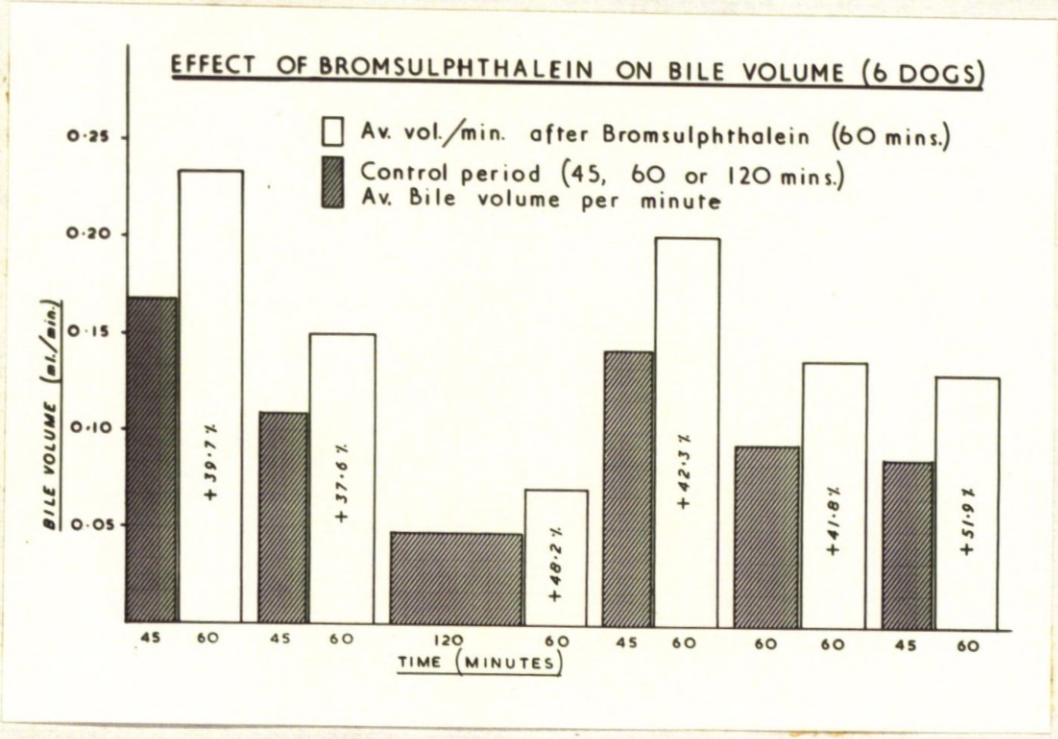
Figure 46 shows the effect of bromsulphthalein on bile volume in six dogs. The bile volume rate (millilitres per minute) and the average volume per minute after bromsulphthalein has been given, is compared with the control period of observation which ranged from 45 to 120 minutes in these 6 dogs. The respective time of the control observation periods are given. The percentage increase in the bile volume rate is also indicated in each column.

Figure 46

The Effect of Bromsulphthalein on Bile Volume in Six Dogs

The bile volume in millilitres per minute during a control period which varied from 45 - 120 minutes compared with the average bile volume rate after an intravenous injection of bromsulphthalein.

The time period of observation after bromsulphthalein has been 60 minutes in each case. The percentage increase in bile volume (mls./min.) ranges from 37.6% to 51.9%. The average increase in these six dogs being 43.58%.


EFFECT OF BROMSULPHTHALEIN ON BILE VOLUME (6 DOGS)
Av. vol./min. after Bromsulphthalein (60 mins.)
Control period (45, 60 or 120 mins.)
Av. Bile volume per minute
BILE VOLUME (ml./min.)
0·25
0·20
0·15
0·10
0·05
+39·7 %
+37·6 %
+48·2 %
+42·3 %
+41·8 %
+51·9 %
45
60
45
60
120
60
45
60
60
60
45
60
TIME (MINUTES)

It can be seen that the least effect occurred in the second dog, there being only a 37.6 percentage increase in bile volume flow. The greatest effect was noted in the sixth dog, where the increase was as much as 51.9%. In these 6 dogs, there has been a definite choleretic effect ranging from 35 to 52% after the standard injection of bromsulphthalein (5 mg./Kg.) during a period of observation lasting 60 minutes. Judging from the experiment on dog 545, this effect would appear to fall off in time and if the period of observation had been 120 minutes instead of 60 minutes, then the percentage increase in bile volume over the whole period would have been considerably less.

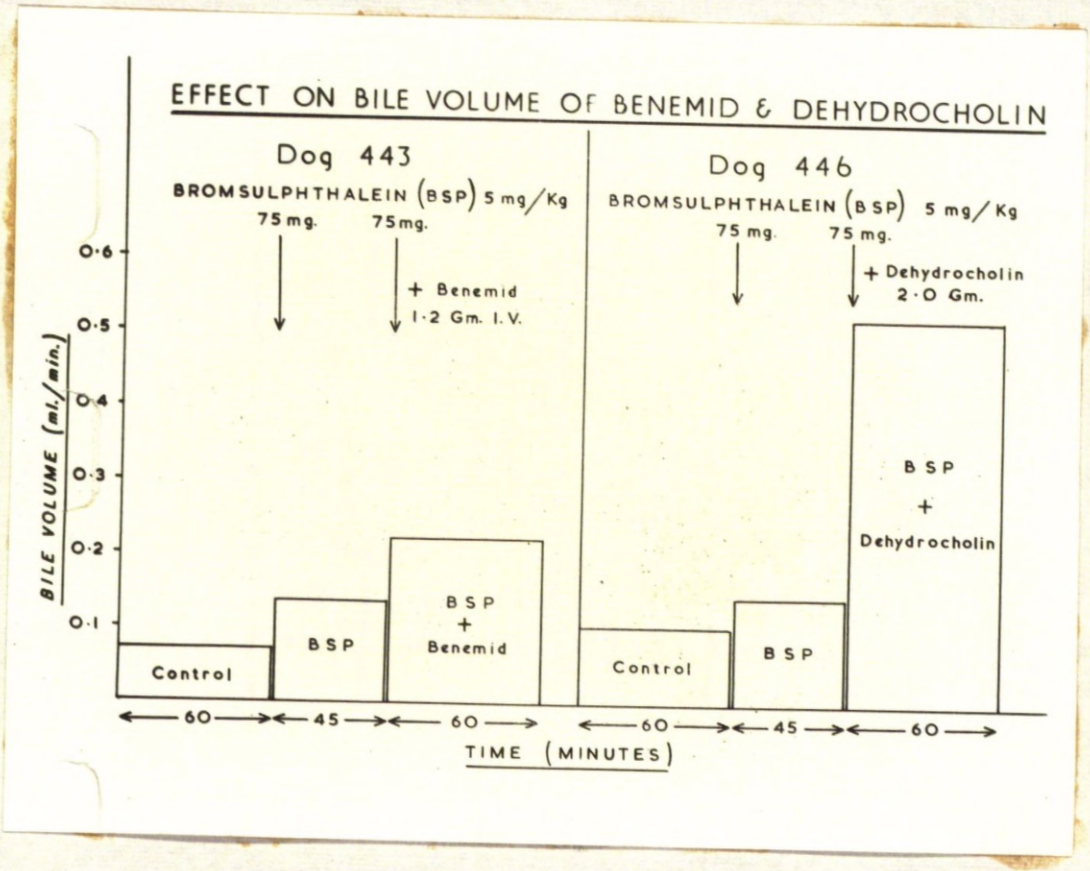
Effect of Benemid on Bile Volume.

The substance probenecid (dye-n-propylsulphamyl benzoic acid; 'Benemid', Merck, Sharpe and Dohme) has been used in later sections of the Results in order to examine the effect of benemid on the hepatic uptake, storage and excretion of bromsulphthalein. Since bromsulphthalein and benemid have been given together it is important to try and determine what effect benemid may have had on the bile volume. Figure 47 is in two parts, showing

Figure 47

The Effect on Bile Volume of Benemid and "Dehydrocolin".

In dog 443, a control period of 60 minutes is compared with the bile volume rate following a 75 mg. dose of bromsulphthalein (5 mg./Kg.) and the observation carried on for a period of 45 minutes, when 75 mg. of bromsulphthalein was given, plus 1.2 gr. Benemid intravenously. The bile volume rate was observed for 60 minutes. There is an increased bile volume following bromsulphthalein alone and again following bromsulphthalein and Benemid together. The later effect is more marked but some of the increase will be due to the extra bromsulphthalein given and some to Benemid. Dog 446 was given 75 mg. bromsulphthalein (5 mg./Kg.) at similar time intervals but on the second injection of bromsulphthalein it was given with 2 gr. "Dehydrocholin". The choleretic effect on this occasion is much more marked and there is a considerable increase in the bile volume rate per minute. In the dose given "Dehydrocholin" has a much greater choleretic effect than Benemid.


EFFECT ON BILE VOLUME OF BENEMID & DEHYDROCHOLIN
Dog 443
BROMSULPHTHALEIN (BSP) 5 mg/Kg
75 mg.
75 mg.
+ Benemid
1·2 Gm. I.V.
Dog 446
BROMSULPHTHALEIN (BSP) 5 mg/Kg
75 mg.
75 mg.
+ Dehydrocholin
2·0 Gm.
BILE VOLUME (ml./min.)
0·6
0·5
0·4
0·3
0·2
0·1
Control
BSP
BSP + Benemid
Control
BSP
BSP + Dehydrocholin
60
45
60
60
45
60
TIME (MINUTES)

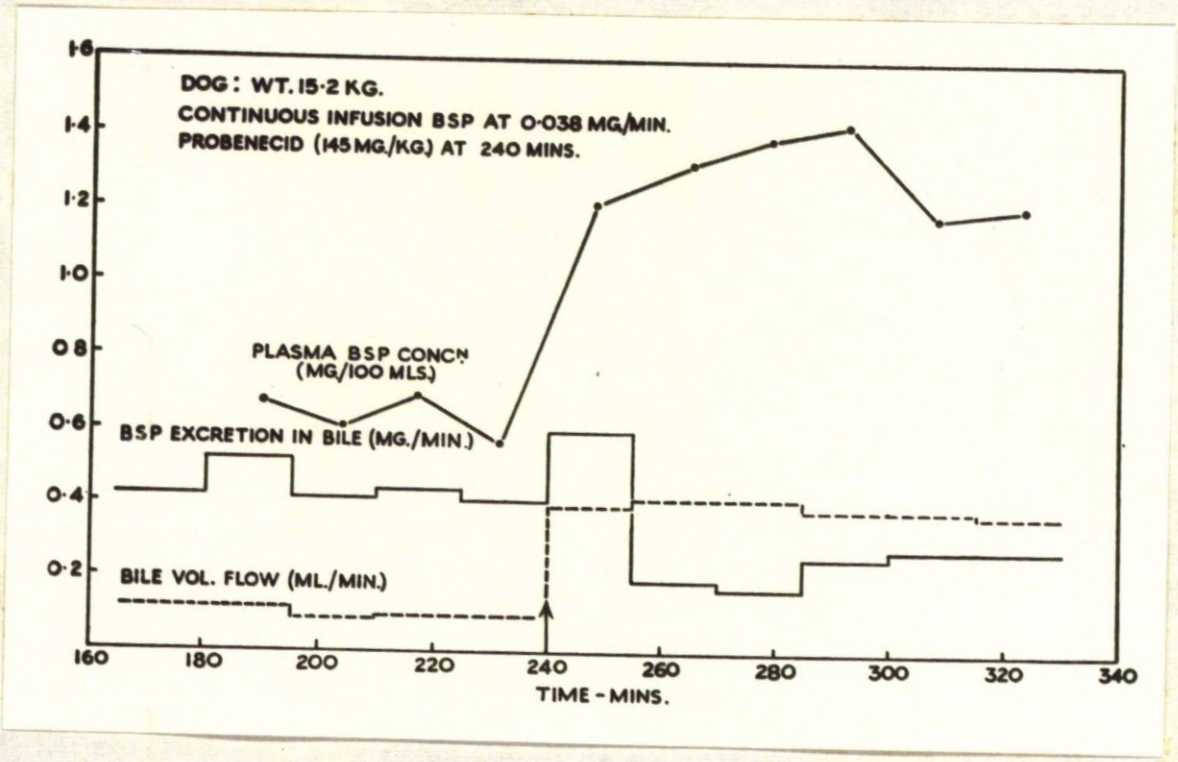
the results of experiments in dogs 443 and 446. In the experiment with dog 443 after an acute biliary fistula was performed, a control period of observation of 60 minutes was carried out and during this time the bile volume rate was 0.071 mls. per minute. Then a 75 mg. dose of Bromsulphthalein was injected intravenously and over the next 45 minutes it can be seen that the bile volume rate increased to 0.138 mls. per minute. At the end of this 45 minute period a further 75 mg. of bromsulphthalein was given plus benemid 1.2 gm. intravenously. During the subsequent 60 minutes the bile volume rate increased to 0.222 mls. per minute. There is no doubt that there is a choleretic effect with bromsulphthalein as compared with the control volume and that some of the increased bile volume rate following the second injection of bromsulphthalein and benemid together will be due to bromsulphthalein alone.

A better example of the effect of benemid on bile volume is in a continuous infusion experiment which is shown in Figure 48. A continuous infusion was given at the rate of 0.038 mg. per minute and an injection of benemid (Probenecid) 145 mg./Kg. was given at two 40 minute intervals. The output of

Figure 48

This shows the effect of Benemid in relation to a continuous infusion of bromsulphthalein. At 240 minutes, Benemid (145 mg./Kg.) was given intravenously.

The Figure shows (i) the rise in plasma bromsulphthalein concentration, (ii) the hydrochoreresis, and (iii) the reduction in biliary bromsulphthalein excretion rate. The ordinate scale applies to all three graphs, the units of which are shown with each graph.


DOG : WT. 15·2 K.G.
CONTINUOUS INFUSION BSP AT 0·038 MG./MIN.
PROBENECID (145 MG./KG.) AT 240 MINS.
PLASMA BSP CONCN (MG./100 MLS.)
BSP EXCRETION IN BILE (MG./MIN.)
BILE VOL. FLOW (ML./MIN.)
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
160
180
200
220
240
260
280
300
320
340
TIME - MINS.

bromsulphthalein in bile during the 75 minutes which preceded the administration of bromsulphthalein varied between 0.525 and 0.413 mg. per minute with a mean of 0.451 mg. per minute. After benemid had been given the rate of bromsulphthalein excretion in the bile in the first 15 minute example rose to 0.6 mg. per minute. This was presumably dye to a "flushing out" of the biliary system of bromsulphthalein already excreted by the liver cells but not yet removed from the bile canaliculi. For the next 75 minutes, the values ranged between 0.197 and 0.28 mg. per minute with a mean rate of 0.254 mg. per minute. The bile volume flow rate in millilitres per minute is indicated by the dotted line on the bottom, where it can be seen that the bile flow rate towards the end of the equilibrium period (165 - 240 minutes) before the administration of benemid varied between 0.093 and 0.12 mls. per minute (with a mean of 0.119 mls. per minute). After the injection of benemid the rate of bile production varied between 0.373 and 0.420 mls. per minute (mean 0.392 mls. per minute). This experiment shows that bromsulphthalein itself produces an increase in the bile volume flow and that this flow is increased still further by benemid, the

increase not being due to any increase in bromsulphthalein excretion because this is, in fact, reduced. Thus, the total result after benemid is presumably a mixed effect, due partly to bromsulphthalein excreted and the benemid.

In a separate series of experiments carried out by Dr. A.E. Goetzee in the same laboratory, he noted that there was a maximum hydrocholeresis which could be produced by benemid. This higher rate of bile volume flow was found to be approximately 2.0 mls. per Kg. per hour and he found that it was rarely possible to exceed this rate under similar conditions to the continuous infusion experiment described. The proportional increase due to benemid alone was found to be determined by the control bile volume flow rate. This can be illustrated by two animals in Dr. Goetzee's series, each of which received benemid in a dose of 150 mg./Kg. and no bromsulphthalein. The control rate in the first was 0.25 mls./Kg./hour, which was increased to 1.53 mls./Kg./hour, an increase of 6.0 times. A control rate in the second dog was 0.52 mls./Kg./hour, increasing to 2.02 mls./Kg./hour, an increase of 3.9 times.

The Effect of Dehydrocholin on Bile Volume

Sodium Dehydrocholate, "Dehydrocholin" (British Drug Houses Ltd.) is the name adopted for the British Drug Houses preparation of dehydrocholim acid (3: 7: 12-triketocholanic acid) a powerful choleretic of low toxicity. This substance has also been used in subsequent sections to compare its effect when given with bromsulphthalein. Figure 46 shows the result of an experiment on an acute biliary fistula (dog 446), in which, during a control period of 60 minutes the bile volume flow rate was 0.105 mls./ minute. After a 75 mg. dose of bromsulphthalein, this increased to 0.146 mls./ minute over the next 45 minutes and then a further 75 mg. of bromsulphthalein plus 2 gms. of "Deyhdrocholin" was injected intravenously. As a result, the bile volume rate per minute increased considerably and the flow rate over the next 60 minutes was 0.523 mls./ minute. Even if the effect of the increase of bile volume following bromsulphthalein and benemid given together in the case of dog 443 was accepted as being due to bromsulphthalein alone, there is no doubt that dehydrocholin has a considerable choleretic effect and it can be seen that in the dose

given, it is certainly a more powerful choleretic substance than bromsulphthalein or benemid or both given together.

The Effect of Biligrafin on Bile Volume

A series of experiments is described later on in the Results in which biligrafin was given at various times before and after bromsulphthalein. Biligrafin is the methylglucaminate of N, N-Adipic-Di (3-Amino-2: 4: 6-Triiodopenzoic acid). This substance is used for radiographic investigations of the biliary system when the gall bladder and bile ducts can be outlined. Since in the literature provided with this substance no mention is made of any effect on bile volume, an experiment was carried out in order to determine its effect. The control volume of bile in an acute biliary fistula dog was noted and then a dose of Biligrafin Forte (1 cc./Kg./ body weight) was given intravenously. This dog was given 12 ccs. of biligrafin and the bile collected at various time intervals. Figure 48 shows that the bile volume (mls.) was increased. In the control period from 30 minutes to 61 minutes prior to the injection of biligrafin, a volume of 5.6 mls. was obtained, whereas in the 15 minute periods following

Figure 49

The Effect of Biligrafin on Bile Volume

The control volume of bile is given for a period of 65 minutes, following which a dose of Biligrafin Forte, 1 cc./Kg./body weight (i.e. 12 ccs.) has been given intravenously.
The increase in bile volume is not very noticeable when the volumes are given in mls. per time period of observation.
This Figure should be compared with Figure 50.

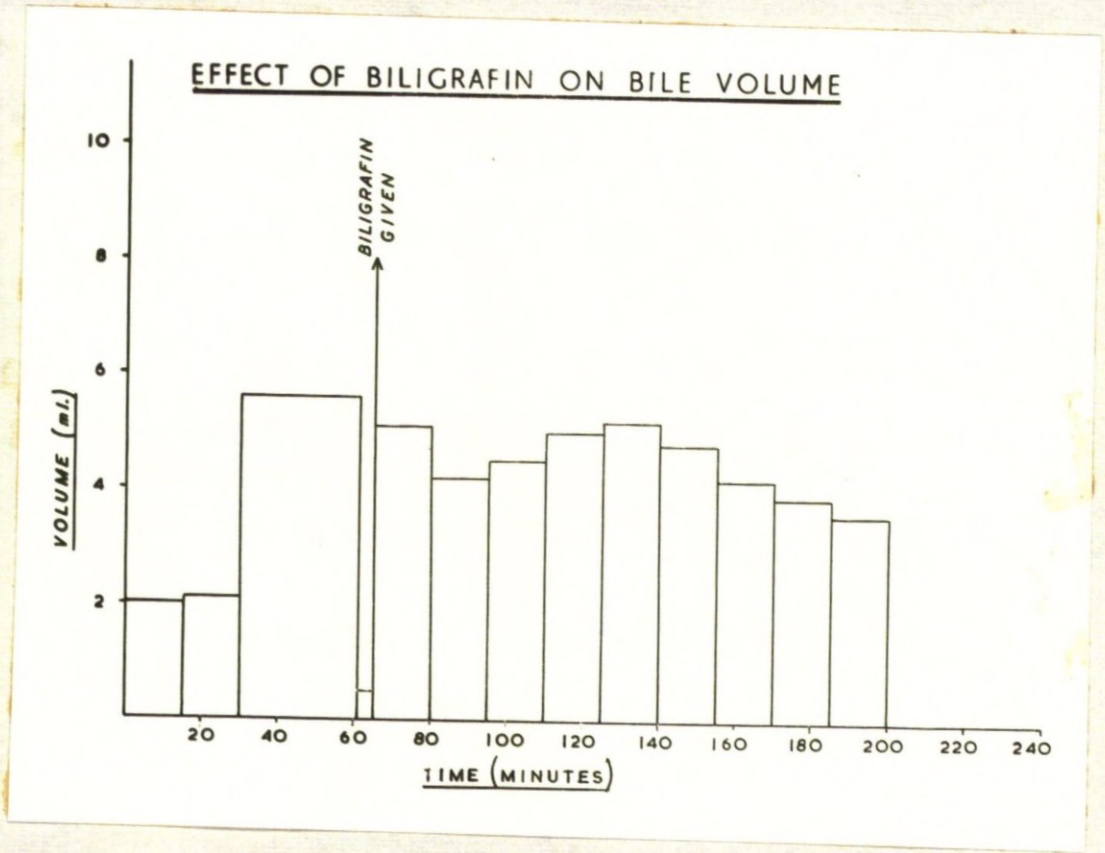
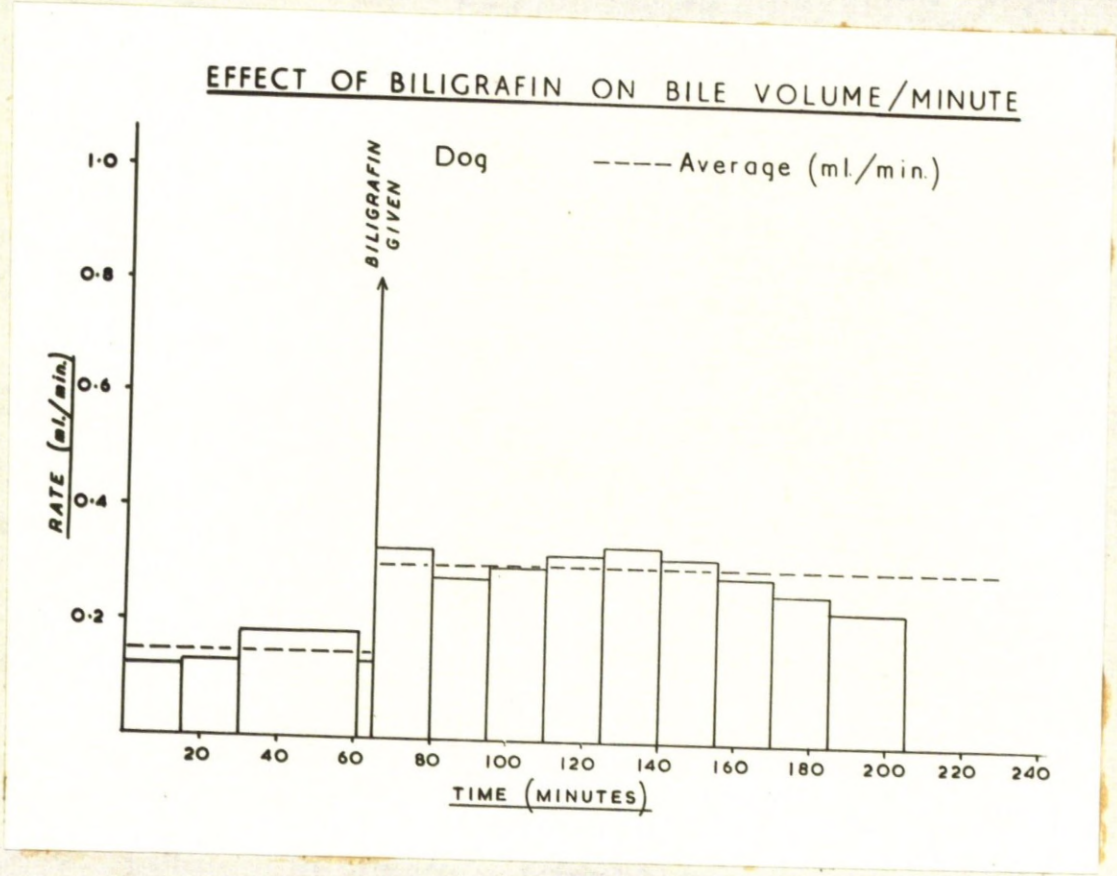

EFFECT OF BILIGRAFIN ON BILE VOLUME
BILIGRAFIN GIVEN
VOLUME (mL)
10
8
6
4
2
20
40
60
80
100
120
140
160
180
200
220
240
TIME (MINUTES)

Figure 50

The Effect of Biligrafin on Bile Volume in mls. per minute

This Figure shows the choleretic effect of biligrafin to a more marked degree than in Figure 49.

The control of bile volume rate in millilitres per minute is given and the average volume during the whole of the control period is indicated by a dotted line. Following the intravenous injection of Biligrafin Forte (12 ccs.) the bile volume rate is noted to be increased by approximately 100%. The average bile volume rate in millilitres per minute is given as a dotted line during the period of observation, following the injection of biligrafin.


EFFECT OF BILIGRAFIN ON BILE VOLUME/MINUTE
Dog
---- Average (ml./min.)
BILIGRAFIN GIVEN
RATE (ml./min.)
1·0
0·8
0·6
0·4
0·2
20
40
60
80
100
120
140
160
180
200
220
240
TIME (MINUTES)

the injection of biligrafin, the bile volume was between 4 and 5 mls. When the bile volume rate (mls. per minute) is plotted, this effect is more apparent. During the control period, the average bile volume rate was 0.149 mls. per minute, which is shown in the dotted line prior to the injection of biligrafin in Figure 49, and the average volume following the injection of biligrafin was 0.301 mls. per minute, again shown as a dotted line. An arrow indicates the time of injection of the biligrafin. There is virtually a two-fold increase in the control bile volume rate following biligrafin in the doses given.

Discussion.

Cook, Beach, Bianchi, Hambourger and Green (1950) have given a value of 0.26 plus or minus 0.024 mls./Kg. per hour as the usual rate of bile flow in anaesthetised dogs. In the experiments which have been carried out through this project of research, the rate of bile production has been noted to be higher than this. In 10 dogs, whose bile was collected and measured before the injection of either bromsulphthalein or any other substance, the mean

Table 12

The increase in bile volume after benemid. In column 6 are shown the percentage increases. These are not proportional to the dose given, but depend largely on the initial bile flow. Where this is small (e.g. experiments 5, 6, 8 and 10) the proportionate increase after benemid is large; where the initial volume is greater (e.g. experiments 4, 9 and 13) the proportionate increase is small.

Bile Volume
ml/Kg/hr.

Dog Bile Volume Series	Weight Kg.	Benemid dose mg/Kg.	Before Benemid	After Benemid	$\frac{\text{After Benemid}}{\text{Before Benemid}} \times 100$
1	18.5	10.0	0.395	0.410	104
		30.0	0.410	0.680	166
2	10.9	100.0	0.597	1.740	292
3	15.2	145	0.470	1.550	330
4	13.2	151.5	0.674	1.290	191
5	17.0	167.5	0.335	1.300	388
6	10.6	94.5	0.317	1.735	548
7	16.2	147.0	0.460	1.540	335
8	20.0	125.0	0.357	1.530	428
9	9.0	83.25	0.727	1.110	151
10	10.0	150.0	0.336	1.650	490
11	9.4	138.0	0.495	1.060	215
12	8.6	174.0	0.551	2.380	432
13	8.2	146.0	0.938	1.840	196
14	8.6	157.0	0.801	2.700	336

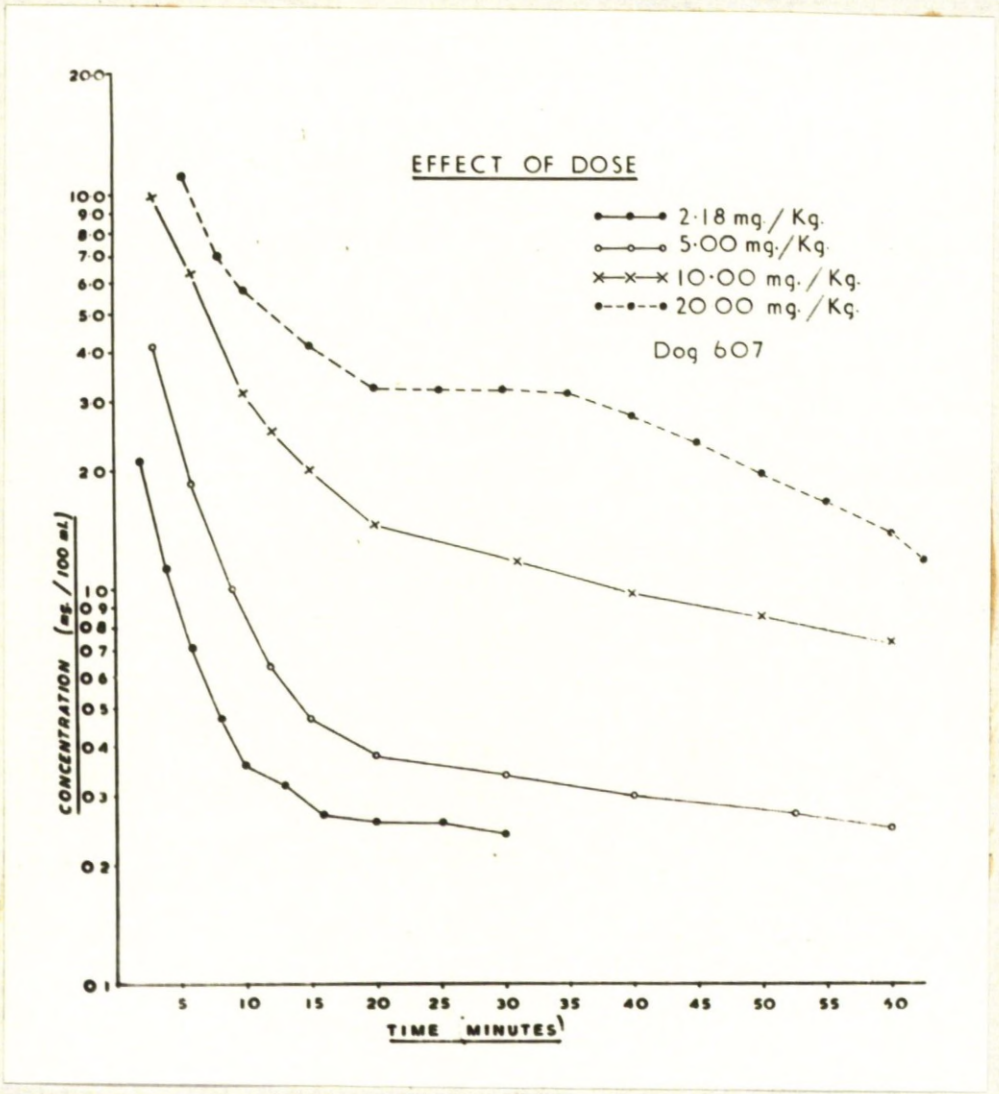
value for bile production was 0.38 mls./Kg. per hour (the range was 0.20 - 0.54). The disparity between this value and that given by Cook et al (1950) may be due to the differences in diets of the animal prior to the experiment. As mentioned previously, the effect of bromsulphthalein itself on bile volume has not been commented on, with the exception of Sperber (1957). The specific effect of bromsulphthalein om bile volume is shown in the results in this Section. Of the other substances used in this work, benemid has been shown by Goetzee, Richards and Tindall, 1960, to have a definite choleretic effect. Table 12 shows the results of a series of continuous intravenous infusion experiments by these workers which shows the increase in bile volume flow after benemid. "Dehydrocholin" is a well-known choleretic agent and being used in clinical practice for this particular purpose. Biligrafin, as far as one can tell, has not been specifically investigated for its effect on bile volume, presumably because its main use is as an X-ray contrast agent for intravenous cholesyst-angiography. This substance contains a high iodine content, the iodine content of the acid product

being 66.9%. The iodine content of the salt is 50%. One ampoule of Biligrafin contains 20 ccs. of a 30% solution (that is 6 grammes of active agent) and one ampoule of Biligrafin Forte, which has been used in this work, contains 20 ccs. of a 50% solution, i.e. 10 grammes of active agent. It has been shown in the dog that it has a specific choleretic effect, increasing the bile volume in the example given by 100%. The effect of benemid, "Dehydrocholin" and biligrafin on the disappearance of bromsulphthalein from the circulation when challenged with these substances, is considered in a later Section, so it was felt advisable to determine the effect on bile flow separately.

C. FACTORS AFFECTING THE BROMSULPHTHALEIN DISAPPEARANCE CURVE

Figure 51

This Figure shows the effects of varying doses of bromsulphthalein given in the same animal. This animal was given respectively (at different times with a delay of a week or more between each experiment) doses of 2.18 mg. 5 mg. 10 mg. and 20 mg. The observed plasma concentrations at each corresponding time are elevated as compared with those obtained using a lower dosage. In the case of the 20 mg. dose, there is a phase where there appears to be an almost straight line existing between 20 - 35 minutes, and this may possibly be due to the maximum excretory capacity of the liver to excrete bromsulphthalein in this particular animal having been reached.


EFFECT OF DOSE
2·18 mg./Kg.
5·00 mg./Kg.
10·00 mg./Kg.
20·00 mg./Kg.
Dog 607
CONCENTRATION (mg./100 ml.)
TIME MINUTES
200
100
90
80
70
60
50
40
30
20
10
09
08
07
06
05
04
03
02
01
5
10
15
20
25
30
35
40
45
50
55
60

The Effect of Dose

The standard dose of bromsulphthalein given has usually been 5 mg./Kg. of body weight. In a later Section (E), in which the effect of various substances on the bromsulphthalein disappearance curve are described, it can be seen that when these substances are given at varying time intervals after the injection of bromsulphthalein, the observed concentrations approximate very closely to the control curve, up to the time that the competitive substance is injected. It was noted several times that the typical disappearance curve of bromsulphthalein is readily reproducible in the same animal, given the standard dose. Figure 51 compares the effect of various doses of bromsulphthalein given in the same dog on different dates. The effect of a 2.18 mg./Kg. dose and a 10 mg./Kg. dose of bromsulphthalein has been given. There is an alteration in the initial slope in all of these graphs when compared with each other, but this is presumably due to the fact that only one or two observations have, in fact, been obtained during the initial phase and the subsequent points appear on the "bend" of the normal disappearance curve. The impression is that the

slope of the second portion is approximately the same. The dotted curve, when the animal was given 20 mg. of bromsulphthalein approximates almost to a straight line between 20 and 35 minutes, and it is possible that in this dog, the maximum excretory capacity of the liver for bromsulphthalein had been approached or reached. It could not, however, be confirmed, as the animal was examined "intact". An acute biliary fistula operation was not performed.

The effect of Oestrus ("Heat")

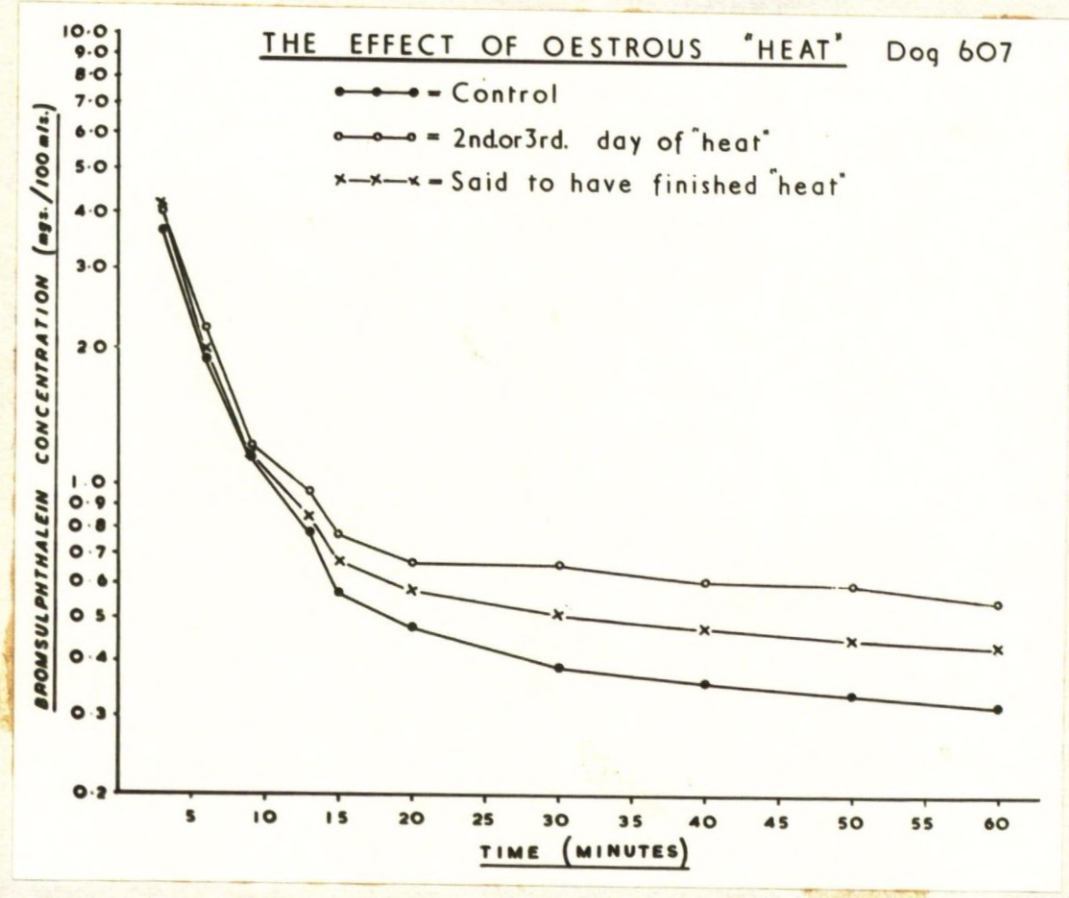
Dog 607 which was used to test the effect of dose was a female and during her stay in the Animal House was noted to be on the second or third day of "heat". The observed plasma concentrations when the animal was on "heat" are at a higher level than those which have been observed with the control curve obtained 2 weeks before "heat" took place (Figure 52). The interesting point is that the slope of the second phase appears to approximate to that of the control curve, but is at a persistently higher level. A curve taken when the animal was thought to have finished "heat" was in fact between these two curves, with the

Figure 52

This graph shows the effect of Oestrus ("Heat") in dog 607.

A control graph is compared with (i) the disappearance curve obtained on the second or third day of "heat" and (ii) the curve obtained when the animal was thought to have finished heat, but in fact the systemic effects judged by the graph obtained did not appear to have completely settled.

In each case a 5 mg./Kg. dose of bromsulphthalein was given.


THE EFFECT OF OESTROUS "HEAT"
Dog 607
●—●—● = Control
○—○—○ = 2nd.or3rd. day of "heat"
×—×—× = Said to have finished "heat"
BROMSULPHTHALEIN CONCENTRATION (mgs./100 c.c.)
10·0
9·0
8·0
7·0
6·0
5·0
4·0
3·0
2·0
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
5
10
15
20
25
30
35
40
45
50
55
60
TIME (MINUTES)

initial readings corresponding closely to that of the control curve. The animal, however, was noted to have a slightly raised body temperature as compared with the normal control state and it was considered that the dog had not quite recovered from the effects of oestrus. It is difficult to be certain exactly why the difference occurs with the animal on heat but it may well be due to the progestational effect on the circulation as a whole, or it could merely be due to the fact that the animal, during this time, had a slightly elevated temperature. The initial phase on all occasions is well within the normal accepted limit for the intact dogs, as judged by the "scattergram" given in Figure 39. The disappearance curve obtained on the second or third day of "heat" is definitely above the normal limits in the second slower phase of the graph. The last two readings of the middle curve, when the dog was thought to have finished, but probably had not completely recovered from the effects of heat, are also above normal accepted limits.

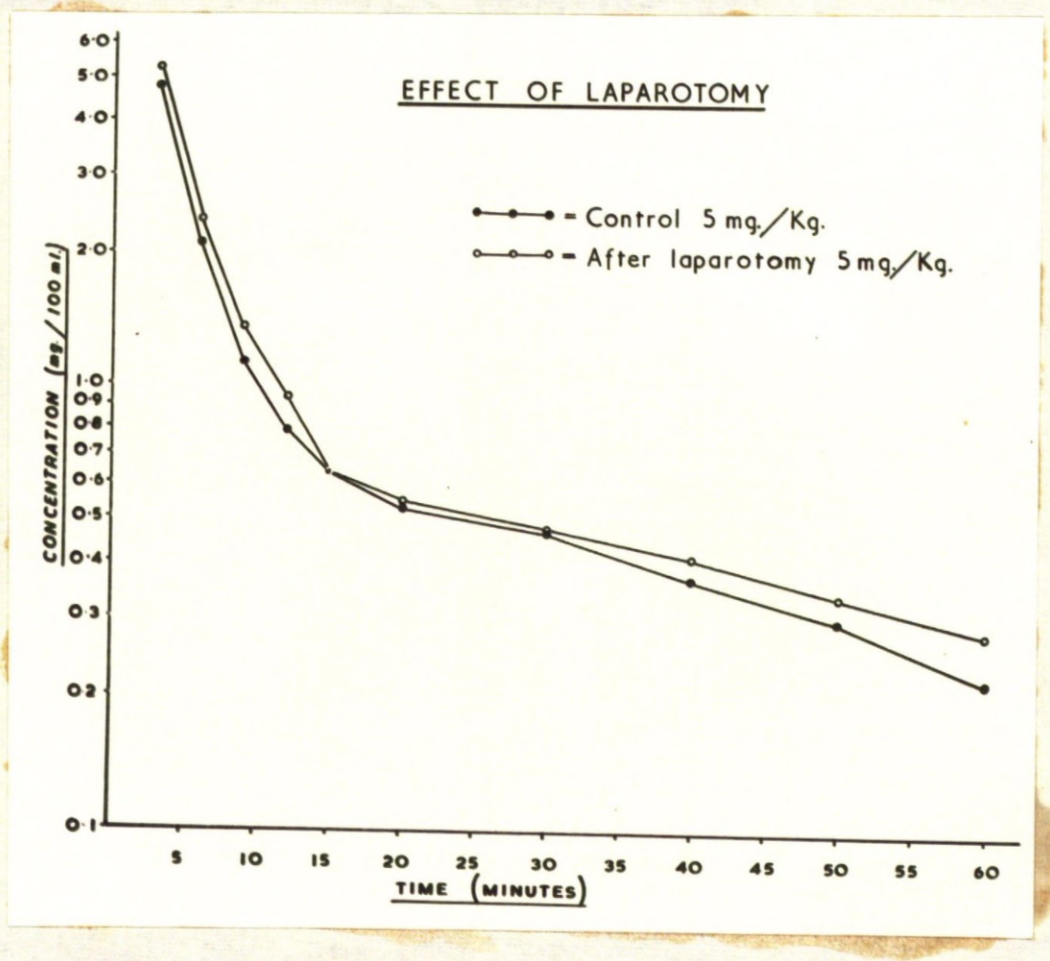
The Effect of Operations

Figure 53 shows the disappearance curves obtained in an animal who had been subjected to a

Figure 53

This depicts the effects of a laparotomy on a dog in which a normal intact control curve obtained one week previously, is compared with that obtained after a laparotomy.

In each case, a 5 mg./Kg. dose of bromsulphthalein was given and it can be seen that the curves correspond well within experimental error.

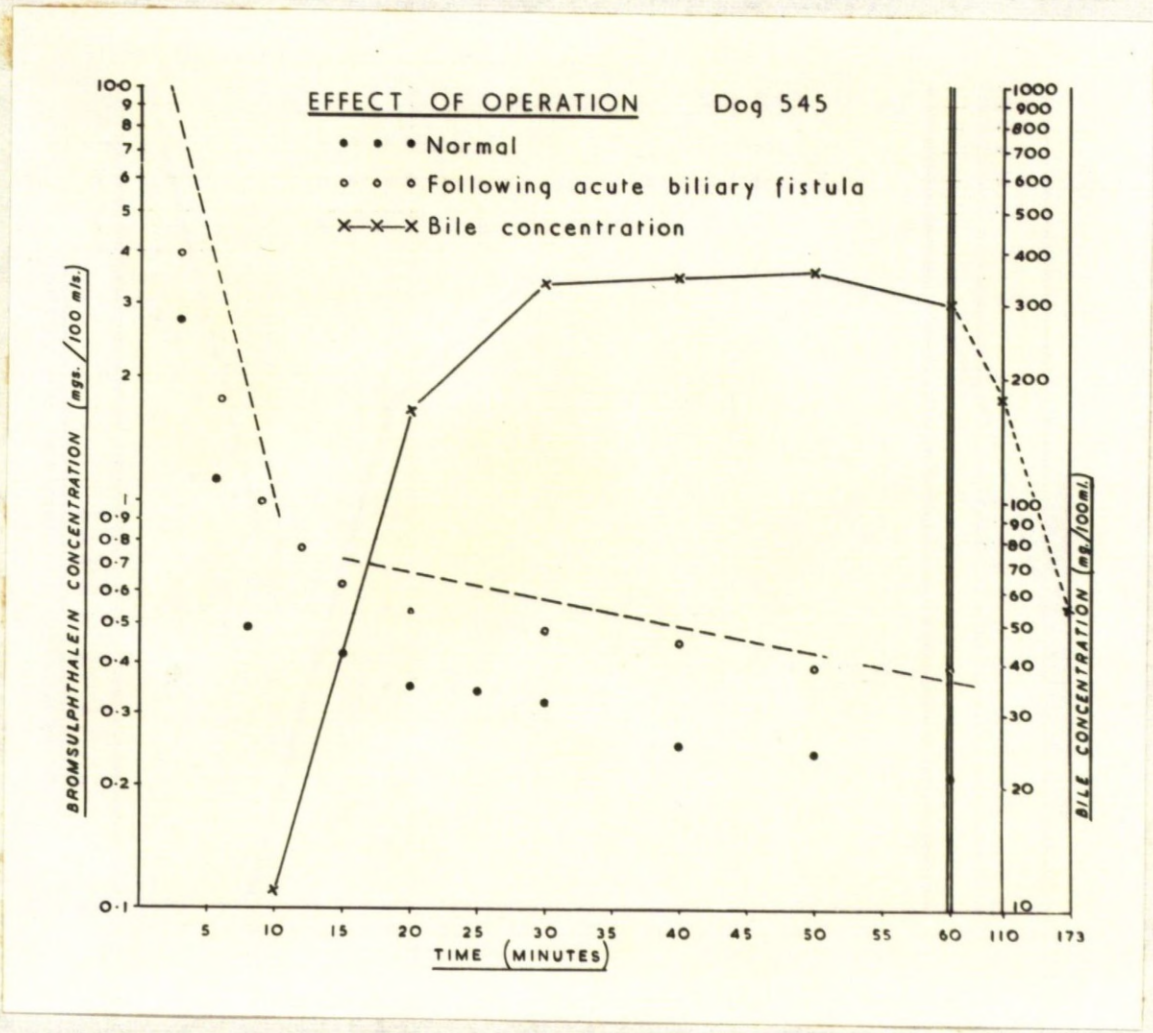

EFFECT OF LAPAROTOMY
●—●—● = Control 5 mg./Kg.
○—○—○ = After laparotomy 5 mg./Kg.
CONCENTRATION (mg./100 ml.)
6·0
5·0
4·0
3·0
2·0
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
5
10
15
20
25
30
35
40
45
50
55
60
TIME (MINUTES)

laparotomy operation only. In this particular case, the abdominal wound was incised as if an acute biliary fistula operation was going to be performed but once the peritoneal cavity had been opened and the gall bladder exposed, the abdominal wound was closed and a bromsulphthalein disappearance curve obtained. There is very little difference between the two disappearance curves in which a standard dose, 5 mg./Kg. body weight was given, and the control curve had been obtained one week prior to the laparotomy operation.

The effect of an acute biliary fistula operation is shown in Figure 54. The normal curve obtained one week prior to the acute biliary fistula operation is shown as well as the dotted lines which represent the outside limits obtained in the "scattergram" of 18 normal intact dogs, shown in Figure 39. The observed plasma concentrations following the acute biliary fistula operation, are at all times higher than those in the normal "intact" state. In dog 545, it is to be noted that the observed concentrations, even following the acute biliary fistula, fall within the normal "scattergram" range, but compared with its control curve, they are elevated at

Figure 54

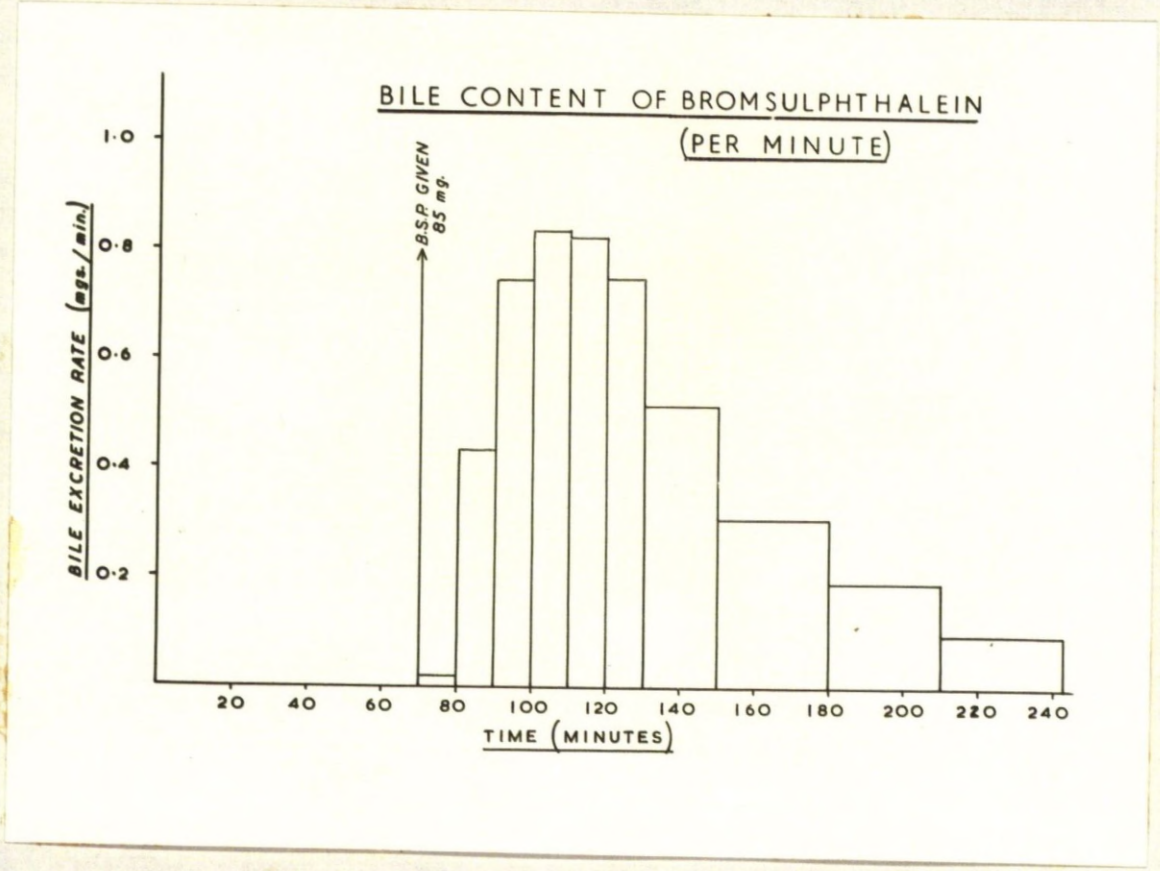
This Figure shows the effect of an operation on dog 545, the operation being an acute biliary fistula. The normal limits are also depicted and it can be seen that although the plasma concentrations are elevated following an acute biliary fistula operation, the disappearance curve in this dog remains within normal limits.

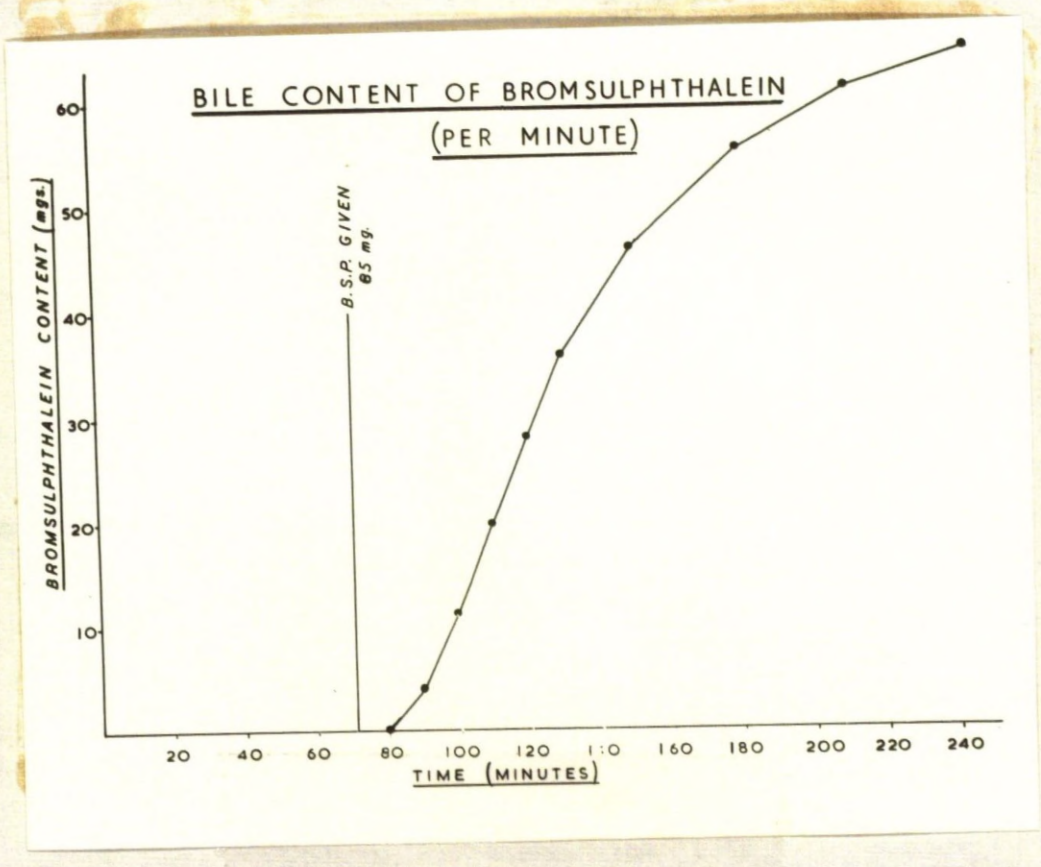

EFFECT OF OPERATION
Dog 545
• • • Normal
o o o Following acute biliary fistula
×—×—× Bile concentration
BROMSULPHTHALEIN CONCENTRATION (mgs./100 mls.)
BILE CONCENTRATION (mg./100ml.)
TIME (MINUTES)
100
9
8
7
6
5
4
3
2
1
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
1000
900
800
700
600
500
400
300
200
100
90
80
70
60
50
40
30
20
10
5
10
15
20
25
30
35
40
45
50
55
60
110
173

any given time. Also shown in this Figure is the bile concentration of bromsulphthalein in mg./100 mls. in which the scale is given on the right hand side of the ordinate. It can be seen at 10 minutes that the concentration of bromsulphthalein in bile is low, whereas at 20 minutes it has reached 165 mg./100 mls. of bile. During the period from 30 - 50 minutes, the bile concentration is approximately 350 mg./100 mls., following which it gradually falls so that at the end of one hour it is of the order of 300 mg./ 100 mls. 173 minutes after bromsulphthalein had been given, the bromsulphthalein concentration in bile is 55 mg./100 mls. Figures 55a and 55b show the bile content of bromsulphthalein per minute in dog 545. Figure 55a shows an initial control period, after which bromsulphthalein was given, following which the bile excretion rate of bromsulphthalein in mg./minute increases to a maximum 30 - 40 minutes from the injection of bromsulphthalein and is maintained at a higher rate until 60 minutes have elapsed, when it falls quite rapidly. At the end of the period of observation (173 minutes), there is still just over 0.1 mg. of bromsulphthalein being excreted per minute. Figure 55b shows the bromsulphthalein

Figures 55 (a) and (b)

This Figure compares the bile content of bromsulphthalein in milligrammes per minute in dog 545, following an acute biliary fistula. It indicates the normal pattern of bromsulphthalein excretion following an acute biliary fistula operation.


BILE CONTENT OF BROMSULPHTHALEIN
(PER MINUTE)
B.S.P GIVEN
85 mg.
BILE EXCRETION RATE (mgs./min.)
1·0
0·8
0·6
0·4
0·2
20
40
60
80
100
120
140
160
180
200
220
240
TIME (MINUTES)


BILE CONTENT OF BROMSULPHTHALEIN
(PER MINUTE)
BROMSULPHTHALEIN CONTENT (mgs.)
B.S.P. GIVEN
85 mg.
60
50
40
30
20
10
20
40
60
80
100
120
140
160
180
200
220
240
TIME (MINUTES)

content in mgs. recovered and not per minute, as indicated in the Figure. It can be seen that after a short time delay bromsulphthalein is excreted in the bile. Sixty minutes after the bromsulphthalein had been given, almost 36 mg. of the dye had been recovered (just over 42% of the dye given). At the end of the experiment (173 minutes), just over 64 mg. of bromsulphthalein had been recovered (slightly more than 75% of the dose given). This recovery rate of bromsulphthalein in the bile in this acute biliary fistula animal would appear to fall well within the limits shown in Table 6.

The effect of an acute biliary fistula operation on the bromsulphthalein disappearance curves appears to be of two types. In a number of dogs, the plasma disappearance curve remains within the normal limits of intact animals, although raised above its own control. On other occasions, it will fall outside the normal limit. Figure 56 shows 5 dogs given the standard dose of bromsulphthalein following an acute biliary fistula operation. Some of the observed concentrations fall within the normal limits, but a number fall outside the accepted limits.

Figure 56

This Figure shows the observed plasma concentrations in relation to time in 5 dogs, given a standard dose of bromsulphthalein (5mg./Kg.) following an acute biliary fistula operation. It can be seen that in certain cases the observed concentrations fall well within the "normal" limits but others are outside the normal limits.

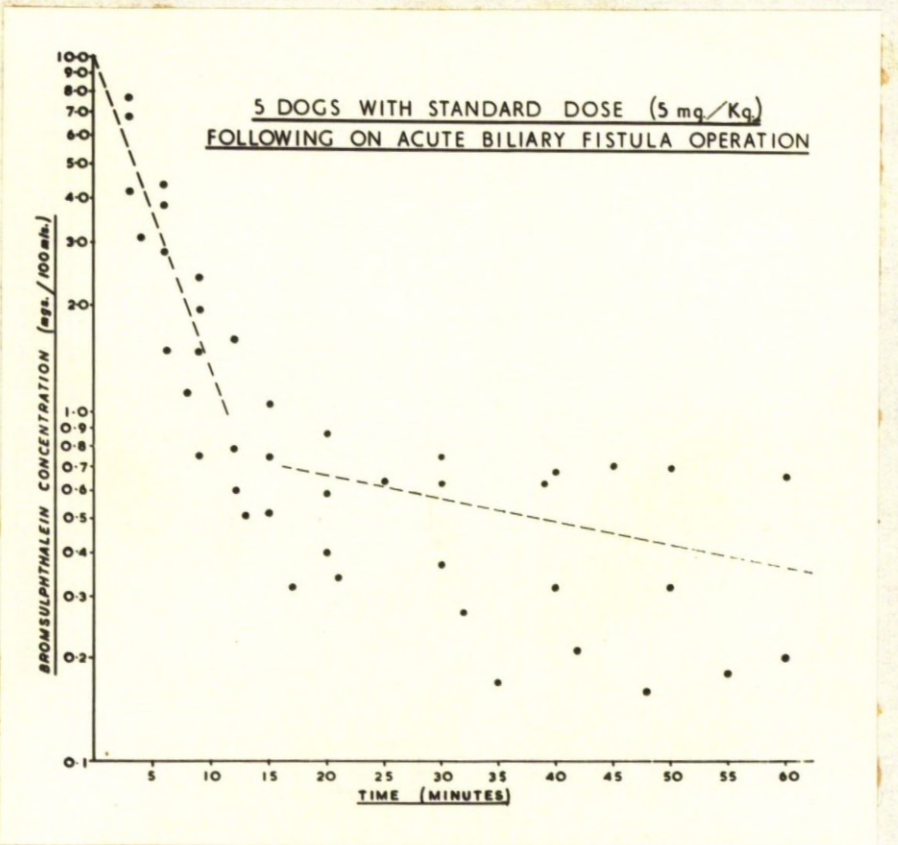

5 DOGS WITH STANDARD DOSE (5 mg/Kg)
FOLLOWING ON ACUTE BILIARY FISTULA OPERATION
BROMSULPHTHALEIN CONCENTRATION (mgs./100 mls.)
100
90
80
70
60
50
40
30
20
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
5
10
15
20
25
30
35
40
45
50
55
60
TIME (MINUTES)

Figure 57

This Figure shows the effect of an operation on dog 10.
The observed plasma concentrations of a normal intact dog are given and the normal scattergram limits are depicted.
It can be seen that the plasma disappearance curve in dog 10 is at all times outside normal limits.

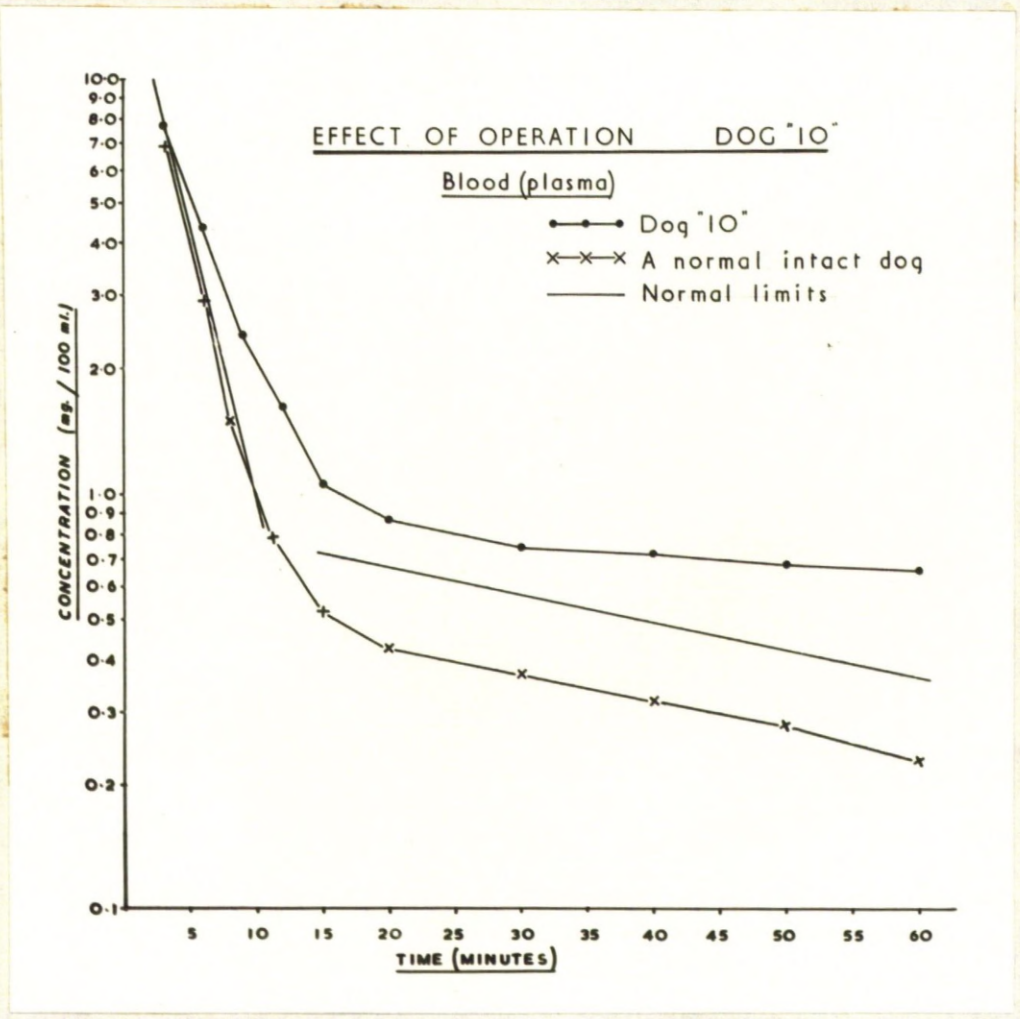
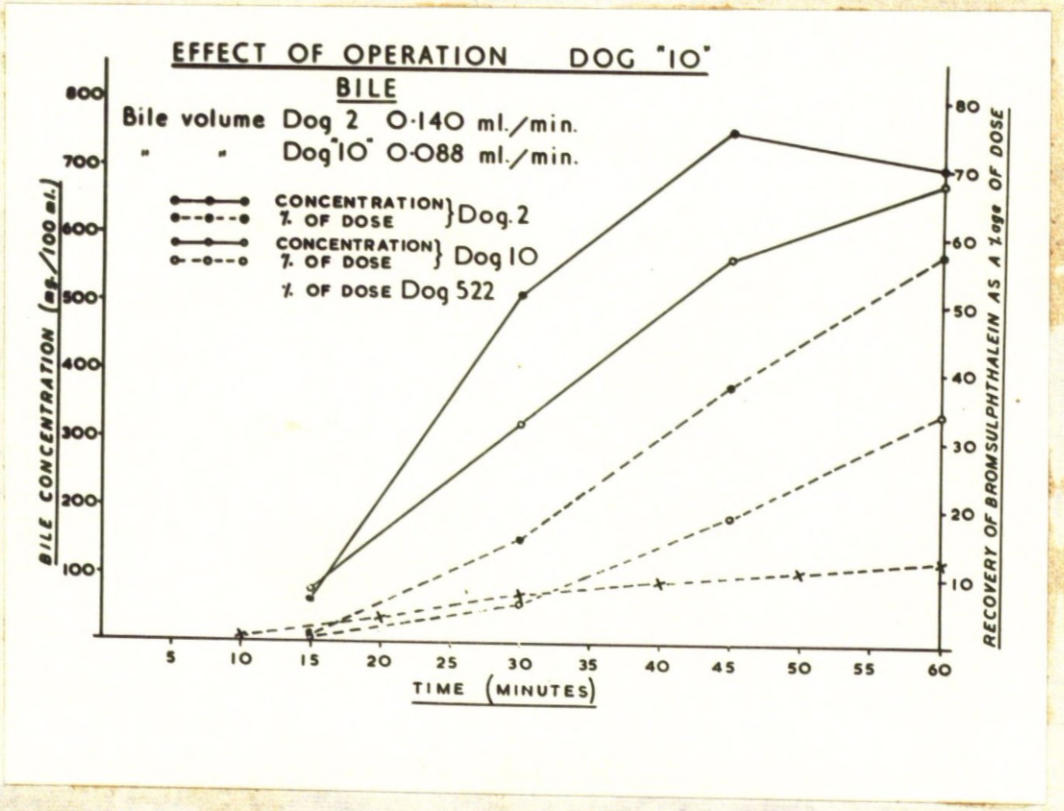

EFFECT OF OPERATION DOG "10"
Blood (plasma)
Dog "10"
A normal intact dog
Normal limits
CONCENTRATION (mg. / 100 ml.)
10·0
9·0
8·0
7·0
6·0
5·0
4·0
3·0
2·0
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
5
10
15
20
25
30
35
40
45
50
55
60
TIME (MINUTES)

Figure 57 shows the effect of an operation on dog 10. Where normal intact dog's plasma disappearance curve and the normal accepted limits are compared with the plasma disappearance curve obtained in dog 10, the results for dog 10 can be seen to fall well outside these limits at all phases.

Figure 58 details some of the effects noted in this animal in more detail. The bile volume noted in dog 2 (in Section A of the Results) had an average of 0.140 mls./ minute, whereas in dog 10 the average volume was 0.088 mls./minute, following the injection of bromsulphthalein. The bile concentration of bromsulphthalein is compared in dog 2 and dog 10 by the solid lines. The actual percentage of the dose of bromsulphthalein recovered is compared in these animals and these are shown as dotted lines. It can be seen that there is less bromsulphthalein recovered, despite the fairly reasonable correspondence of the bile concentration in mg./100 mls. In fact, there is only about half of the bromsulphthalein percentage of dose recovered in dog 10 compared with dog 2, which is considered to be perfectly normal. Also shown in this graph is the percentage of dose recovered in dog 522, who, after a

Figure 58

The Figure shows in more detail some of the effects of operation on dog 10. The bile concentration of bromsulphthalein is indicated in a normal dog (dog 2) and dog 10. The percentage of the dose in each case which has been recovered in relationship to time is given. In the case of dog 10, the recovery of dye is approximately half of that obtained in the normal dog 2. The percentage of the dose which has been recovered in dog 522 which received an intravenous injection of biligrafin 10 minutes after a 5 mg./Kg. dose of bromsulphthalein intravenously. The recovery of dye in dog 522 is even less than that obtained in dog 10.


EFFECT OF OPERATION DOG "10"
BILE
Bile volume Dog 2 0·140 ml./min.
" " Dog "10" 0·088 ml./min.
CONCENTRATION
% OF DOSE
Dog. 2
CONCENTRATION
% OF DOSE
Dog 10
% OF DOSE Dog 522
BILE CONCENTRATION (mg./100 ml.)
RECOVERY OF BROMSULPHTHALEIN AS A % age OF DOSE
TIME (MINUTES)
800
700
600
500
400
300
200
100
80
70
60
50
40
30
20
10
5
10
15
20
25
30
35
40
45
50
55
60

standard dose of bromsulphthalein (5 mg./Kg.) was given biligrafin at 10 minutes. In this animal it is to be noted that there is an even lower percentage recovery of bromsulphthalein.

The effect of interference with the portal venous circulation.

Figure 59 shows the effect of portal vein tie as a two-stage operation carried out on two dogs, 579 and 581. The portal vein tie as a two-stage operation had been completed at least one month prior to the performance of the first test on each dog. It can be seen that in the case of curves 1 and 3, the initial phase appears to be "less rapid" than usual. The second slower phase appears to take place at a normal rate with the exception that curve 3 would be outside the normal accepted limits for an intact animal. The second estimations have taken a further month, that is approximately two months after the completion of portal vein tie operation in each case. In dog 579, the disappearance curve is not within normal limits but this animal had lost 1½ Kgs. in body weight since the previous test. The bromsulphthalein disappearance curve tends to be elevated at all levels with an increasing dose,

Figure 59

The Effect of Portal Vein Ties.

Two dogs (579 and 581) who had the portal vein ligated in a two-stage operation. The second stage of the operation having been completed approximately one month prior to the first curve in each animal being taken. The second plasma disappearance curve in each dog was repeated a month later, i.e. two months after the portal vein circulation had been occluded.

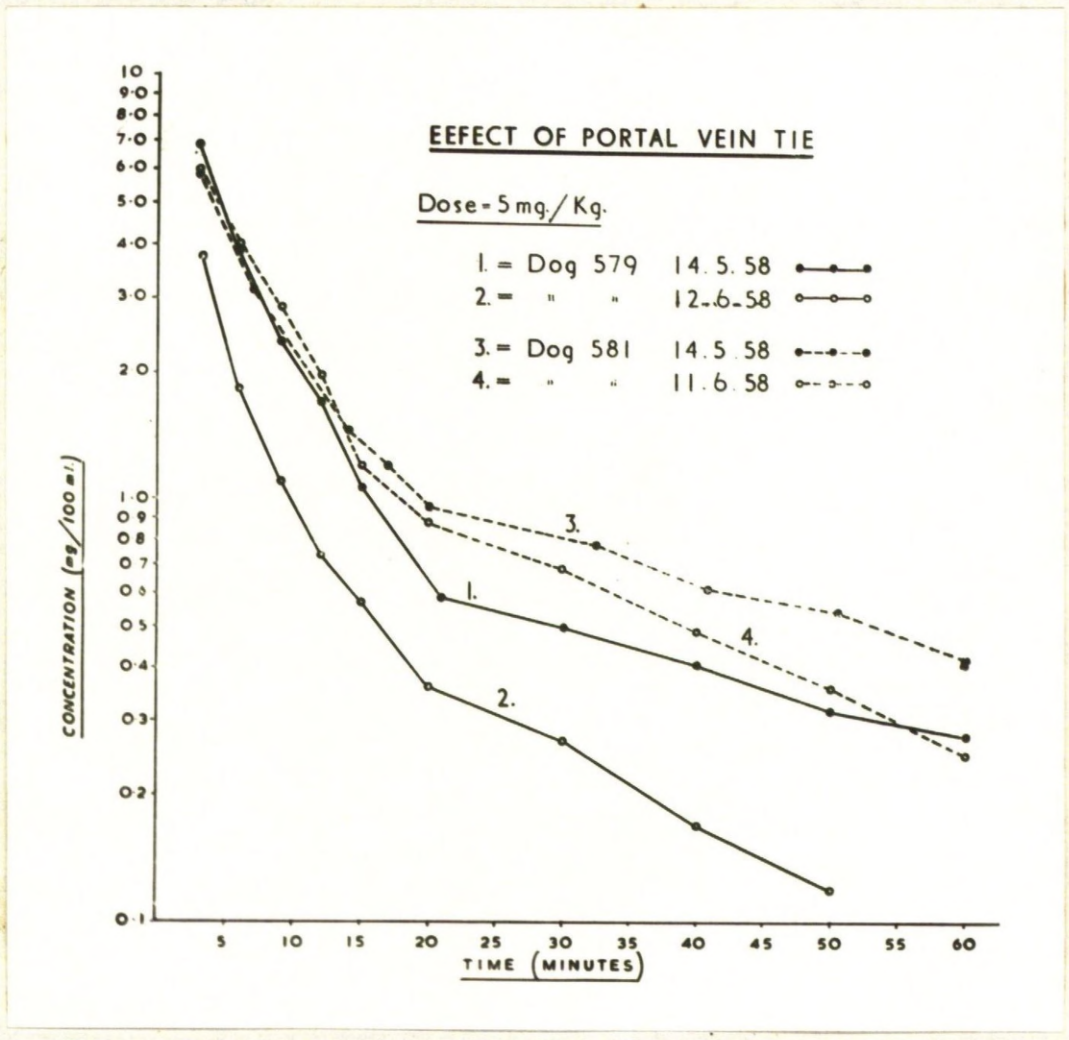
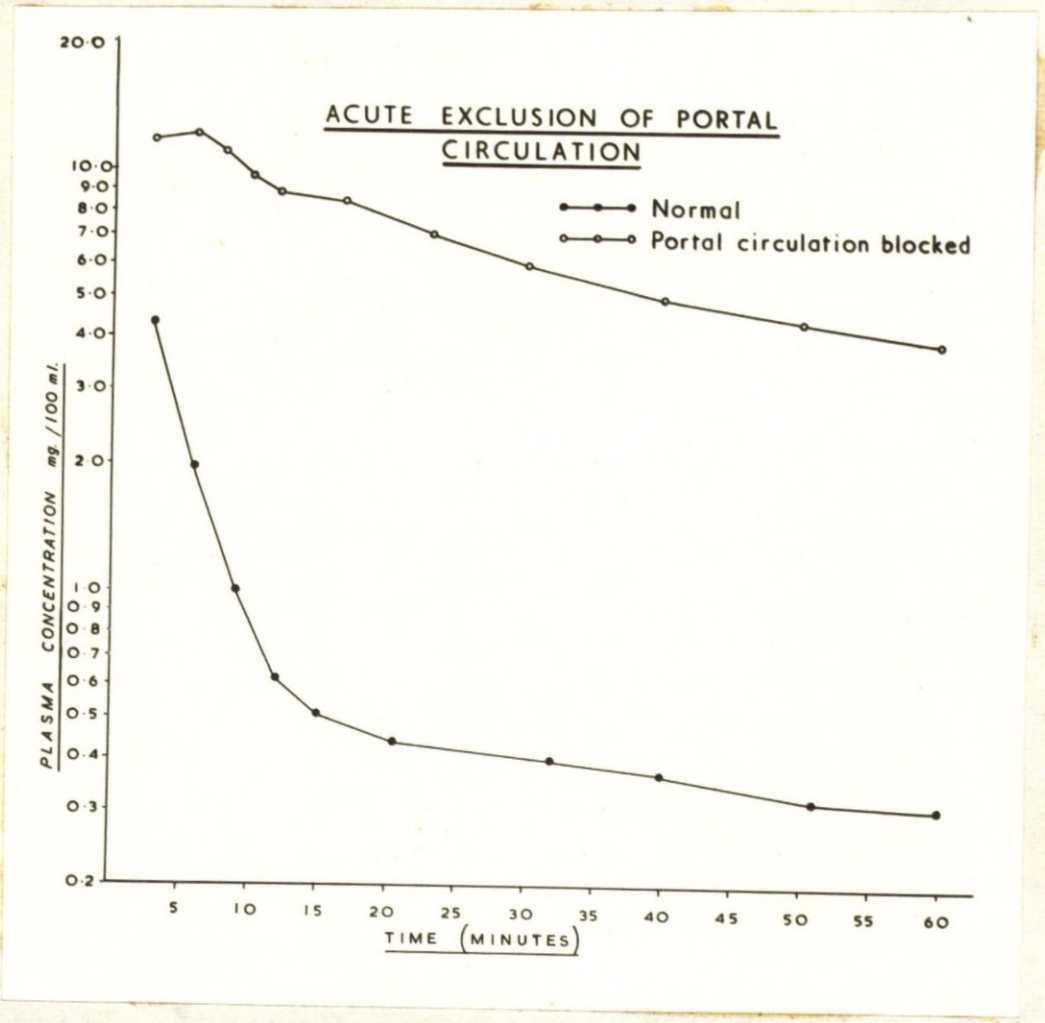

EEFECT OF PORTAL VEIN TIE
Dose - 5 mg./Kg.
1. = Dog 579 14.5.58
2. = " " 12-6-58
3. = Dog 581 14.5.58
4. = " " 11.6.58
CONCENTRATION (mg/100 ml)
TIME (MINUTES)
10
9·0
8·0
7·0
6·0
5·0
4·0
3·0
2·0
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
5
10
15
20
25
30
35
40
45
50
55
60

Figure 60

This depicts the effect of the acute occlusion of portal vein circulation. The normal control curve is given in the animal who, eight days later, had the portal vein ligated as a one-stage operation.


ACUTE EXCLUSION OF PORTAL CIRCULATION
Normal
Portal circulation blocked
PLASMA CONCENTRATION mg./100 ml.
20·0
10·0
9·0
8·0
7·0
6·0
5·0
4·0
3·0
2·0
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
5
10
15
20
25
30
35
40
45
50
55
60
TIME (MINUTES)

and it may well be that the second curve would be much closer to that of the first curve if the original 5 mg./Kg. dose of bromsulphthalein given had been based on the original weight. In the case of the second dog it maintained its weight. Curve 4 can be seen to more closely approximate to curve 3 with the exception that the second slower phase appears to take place more rapidly so that the later observed concentrations fall within normal limits.

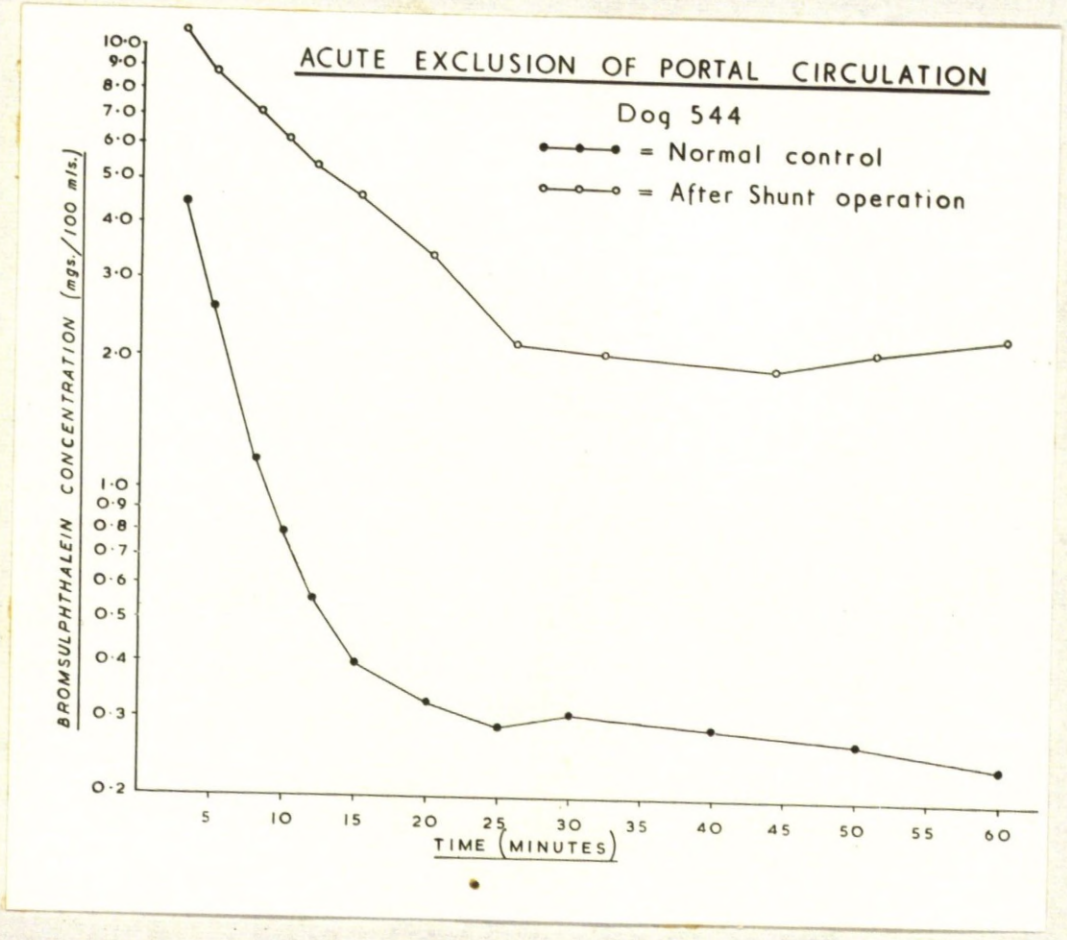
Figure 60 shows the effect of acute exclusion of the portal circulation. In this animal a normal curve was first obtained and eight days later the animal was operated on and the portal vein ligated as a one-stage procedure. Following this, the plasma disappearance curve of bromsulphthalein was determined. It can be seen that the observed plasma concentrations were extremely high so that even at 60 minutes the concentration in the plasma was 4 mg./100 mls. The 3 minute concentration appears to be less than that noted at 6 minutes and this may be due to the mixing time being prolonged under these circumstances. It is difficult graphically to be certain whether or not there is only one phase in this particular animal or there are two phases.

It is possible that the first phase may be unduly prolonged and during the 60 minute period of observation the slope of this curve is altered. In another animal where the portal circulation was interfered with a "shunt" operation was performed. In this animal, dog 544, the portal vein was anastomosed to the right renal vein. Once the animal appeared to have recovered from this operation the animal was given a 5 mg./Kg. dose. The disappearance curve was then compared with the normal curve obtained four days previously (Figure 61). In this case, where presumably there will be less in the way of immediate circulatory effects from the operation, there appears to be two definite phases. The first initial phase being prolonged for approximately twenty minutes, after which a second phase appears to have occurred. After forty-five minutes however, the observed concentrations appear to have increased and although the reason for this is not obvious, it was noted at 40 - 45 minutes that there was a leak occurring from the site of anastomosis and it may well be that there had been an underestimated blood loss. The compensatory circulatory changes may have resulted in the raised plasma concentrations. The general condition of the animal,

Figure 61

This Figure shows the effect of the acute occlusion of the portal circulation by a by-pass ("shunt") operation.

The normal curve is compared with the same animal after a "shunt" operation had been performed. In this case the portal vein was anastomosed with the renal vein.


ACUTE EXCLUSION OF PORTAL CIRCULATION
Dog 544
= Normal control
= After Shunt operation
BROMSULPHTHALEIN CONCENTRATION (mgs./100 mls.)
10·0
9·0
8·0
7·0
6·0
5·0
4·0
3·0
2·0
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
5
10
15
20
25
30
35
40
45
50
55
60
TIME (MINUTES)

however, was quite satisfactory at this time.

The effect of repeated injections of bromsulphthalein

In a few animals, the effect of repeated doses of bromsulphthalein was observed. Figure 62 depicts one such experiment in which the animal was given a 5 mg./Kg. dose of bromsulphthalein and the observed concentrations obtained for a period of 30 minutes. At the end of the 30 minutes a further 5 mg./Kg. dose of bromsulphthalein was given, and the plasma concentrations observed for a further period of 30 minutes, and then again a further injection of bromsulphthalein was given and the plasma concentrations were observed for a period of 30 minutes. It can be seen that the initial graph obtained from the observed concentration shows the typical two components and an initial phase representing the line A - B and the second slower phase represented by the line C - D and the portion which joins the curve between B and C being the so-called "bend". The second curve appears to be similar to that of the first curve as far as the second slower phase is concerned and the slope of these two lines appeared to be identical, that is,

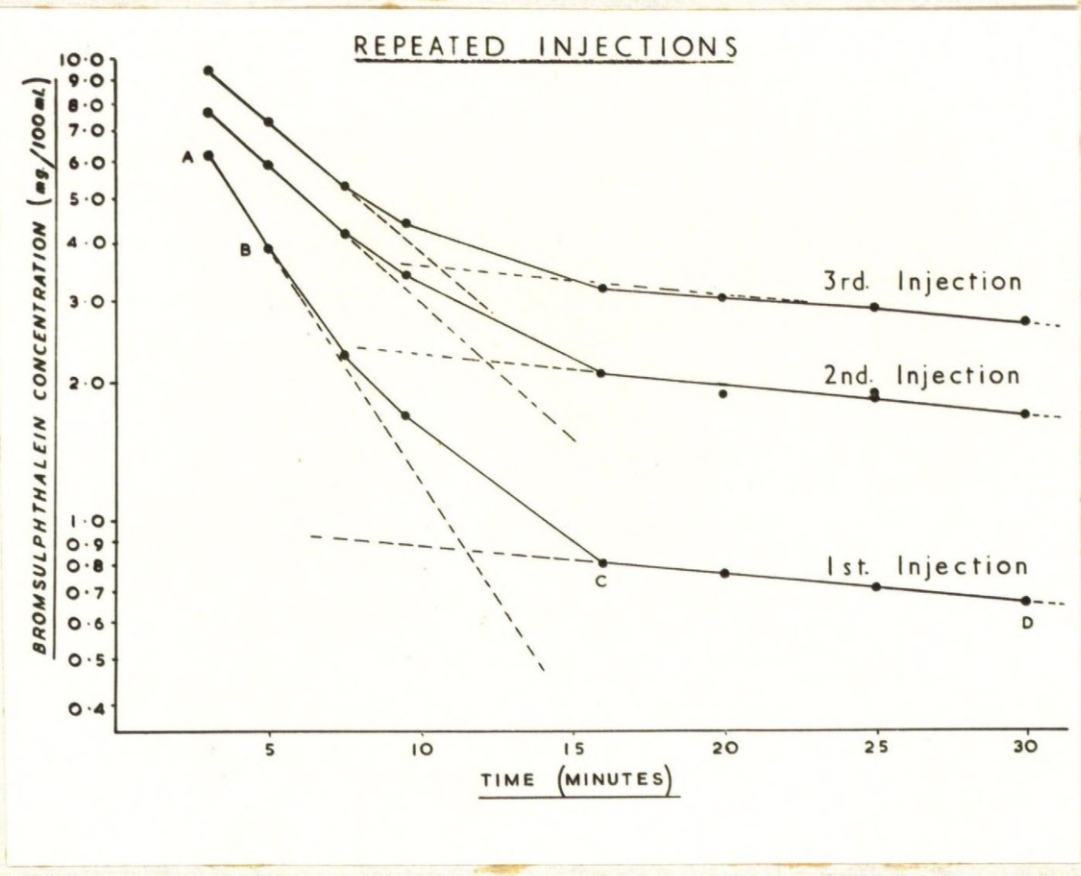
Figure 62

The effect of repeated injection of bromsulphthalein (5 mg./Kg.).

This Figure depicts the plasma disappearance curves following the first, second and third injection of bromsulphthalein; the second injection being given thirty minutes after the first injection and the third injection thirty minutes after the second injection.

The second slower phase, represented by C- D in the first injection disappearance curve, appears to be similar to that which is noted after the second and third injection.

The initial phase A - B, do not appear to correspond to each other.


REPEATED INJECTIONS
BROMSULPHTHALEIN CONCENTRATION (mg/100 ml)
10·0
9·0
8·0
7·0
6·0
5·0
4·0
3·0
2·0
1·0
0·9
0·8
0·7
0·6
0·5
0·4
A
B
C
D
3rd. Injection
2nd. Injection
1st. Injection
5
10
15
20
25
30
TIME (MINUTES)

graphically, these lines appeared to be parallel. The initial phase, however, differs and since the plasma concentrations are obviously higher, it is possible that there are fewer points obtained on the initial phase. Similarly with the third injection, there is an alteration in the slope of the initial phase and the second slower phase again appears to give a line which is parallel to the first and second injections. The mathematical explanation of these graphical results is given in the Appendix. The constants of proportionality for the rates of transfer (i) from blood to liver, (ii) from the liver to blood, and (iii) from liver to bile, namely a, b, h, respectively, are unaltered. Following the first injection, the rate of the rapid phase is so high in the second and third graphs, that the points obtained initially are almost certainly points which would occur on the bend, B - C, of the particular graph concerned. As time proceeds, the second slower phase will have several more observed concentrations so that the line will be better represented. The mathematical reasons for these apparent alterations, despite the rates of proportionality remaining the same, is explained

mathematically in the Appendix.

Discussion

Various factors which may affect the typical bromsulphthalein disappearance curve already noted in the normal intact dog (Section A of the Results) have been studied here. As mentioned previously in the literature, agreement is lacking regarding the optimal dose and times of sampling. In 1939, McDonald suggested that the 5 mg./Kg. dose was most acceptable for clinical purposes because of the increased burden imposed on the liver and the improved chances of testing its ability. The effect of the size of the dose has been determined and it has been noted that the disappearance curves are essentially the same, whatever the dose used, except that the observed concentrations at any time are higher the greater the amount of bromsulphthalein given per Kg./ body weight. On the basis of the mathematical model, the rates of proportionality for a given dog would be the same, the difference being that the load in each direction of transfer would be greater because of the increased dose of dye. At some point, the disappearance curve will be affected because the

liver is unable to deal with more than a certain amount of bromsulphthalein at a given time. In other words, the maximum excretory capacity of the liver for bromsulphthalein will be exceeded. In the case of dog 607 in Figure 51, it was suggested that this level might have been reached since a plateau was obtained in the disappearance curve. If it did occur in the dog at this level then it is difficult to explain exactly why cat 10, who received a very large dose, approximately 63 mg./Kg. body weight, failed to show any such plateau. It is regrettable that further experiments with an increased dosage were not carried out on this particular animal, or indeed in any other animal since it would give a guide as to the maximum excretory capacity of bromsulphthalein when a single intravenous injection of bromsulphthalein is given.

The effect of oestrus ("heat") which was also noted in dog 607 (and this was one of the reasons why further large doses of bromsulphthalein were not given, as the effects of oestrus were observed instead of the increasing size of the dose) oestrus merely happening as an unexpected event in this series of experiments. The raised level of the observed concentrations associated with oestrus may, in fact, be

partially due to the raised body temperature since Brokaw and Penrod (1949) have shown that a fall of temperature has an effect on the rate of disappearance of bromsulphthalein from the plasma. The only experiment in which cooling was tried whilst I was carrying out this Study, was an animal investigated for another purpose and only a couple of blood samples were taken as the animal was cooled to a temperature of 28°C. These isolated concentrations were noted to be lower than would be anticipated in the normal intact animal. One of the effects, apart from the operation in the abnormal dog 10, was due to an increased body temperature. The observed plasma concentrations in this animal were higher than normal. So it is possible that the effects of oestrus are merely due to the raised body temperature, although if this is so, then it clearly reflects a sensitive test due to alterations of temperature which may only be of the order of 0.6 to 1°F.

The effect of operation is important, since the original confirmation of the mathematical model was a comparison of the distribution of dye in the dog, compared with the calculated results, using the

mathematical model and had to be carried out on animals who had had acute biliary fistula operations performed. The effect of a laparotomy, a relatively major procedure in itself, did not appear to affect the bromsulphthalein disappearance curve. Operations on other situations than the abdomen also did not appear to affect the bromsulphthalein disappearance curve. It has also been shown that the effect of performing an acute biliary fistula operation is of two patterns. Firstly that the elevated plasma concentrations, as compared with a normal intact animal, may be within the accepted limits for the normal intact dogs, or a large series of normal intact dogs. Secondly, the observed concentrations in a dog with an acute biliary fistula, may well fall outside the accepted limits due to the fact that the control curve was at the upper limit of the normal range. The observed bile content of bromsulphthalein in animals following an acute biliary fistula, bears a good relationship to that which has been calculated on a basis of a mathematical model alone, as has already been mentioned in Section A of the Results. The most important factor as far as surgery is concerned is that the animal should have

been allowed a reasonable time to recover from the effects of the operation and also that whilst still anaesthetised, it should be maintained in as normal a state as possible, otherwise the effects of the operation may give abnormal results as it did in dog 10, for instance. One of the main things affected in the surgically affected animals is the proportion of the dose of dye which is recovered in the bile in a given time.

The bromsulphthalein disappearance curve obviously depends on two main factors, one, the ability of the liver to take up the dye and, secondly, the ability of the circulation to provide a large quantity of blood to the liver. The effect of interference with this blood supply has been shown to have a marked effect if performed as an acute operation. It is difficult, of course, to say that the effects are predominantly due to the haemodynamic alterations alone, since obviously these operations must have a marked general effect and various compensating mechanisms must be put into action by the animal concerned. A better way of assessing the results would appear to be in the more gradual type

of operation, as indicated by the animals who had the portal vein tied in two stages, since then the liver and body has a chance to adapt itself to the changing internal environment. On the other hand, these animals quite often lose a considerable amount of weight and it is possible that the observed concentrations then are no longer strictly comparable. The effect of repeated injections has been explained mathematically and it is shown that there is no alteration in the animal's ability to deal with bromsulphthalein, but the graphical representation of the results gives an erroneous impression. However, it does mean that it is possible, providing one leaves a longer time interval between the experiments. In one experiment here, Figure 88, and in subsequent experiments carried out by Goetzee (1960) it can be demonstrated that when control curves are obtained over the period of 60 minutes and then one hour allowed to elapse before repeating a further dose of bromsulphthalein, the results are comparable. The curves thus obtained were very similar to those which have been shown for the increased dosage, in other words, the constants characteristic of the graph are very similar to that of the 2.18 mg. and 5 mg./Kg.

dose, shown in Figure 51. The effects of the other substances on bromsulphthalein disappearance curve have, however, been dealt with in subsequent Sections.

D. THE BROMSULPHTHALEIN PLASMA DISAPPEARANCE GRAPHS IN DIFFERENT ANIMAL SPECIES

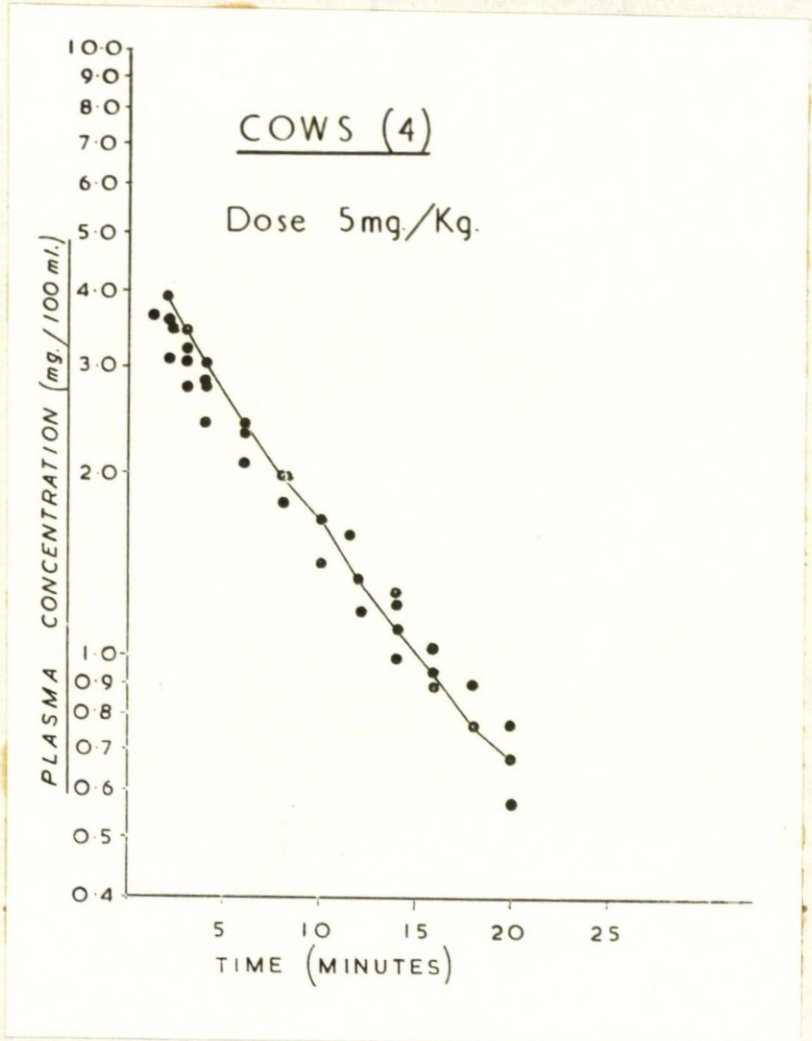
Bromsulphthalein plasma disappearance curves in normal intact cats and dogs have been previously given (Figures 26 and 39 respectively). In the case of the six normal, intact cats the observed plasma concentrations from two to ten minutes approximately, appeared to fall on a straight line, but afterwards the observed concentrations did not fall on the line. It was difficult, however, to confirm this due to the rapid disappearance of dye in the cat, since the observed plasma concentrations after 15 - 20 minutes were at or below the lower limit of estimation, using the colorimetric method described. In one cat (no.10), given a very large dose of bromsulphthalein, the plasma concentrations were on a straight line from 3 minutes to 42 minutes.

The typical bromsulphthalein disappearance curve in dogs has been indicated in the form of a scattergram in Figure 39. Figure 32, which shows the plasma disappearance graph, where it can be seen that the curve follows a typical double exponential curve when the logarithm of the plasma concentration is plotted against time.

The results in other animal species have

Figure 63

The bromsulphthalein disappearance curve in four cows given 5 mg./Kg. body weight.
The logarithm plasma concentration is plotted against time.


COWS (4)
Dose 5mg./Kg.
PLASMA CONCENTRATION (mg./100 ml.)
10·0
9·0
8·0
7·0
6·0
5·0
4·0
3·0
2·0
1·0
0·9
0·8
0·7
0·6
0·5
0·4
5
10
15
20
25
TIME (MINUTES)

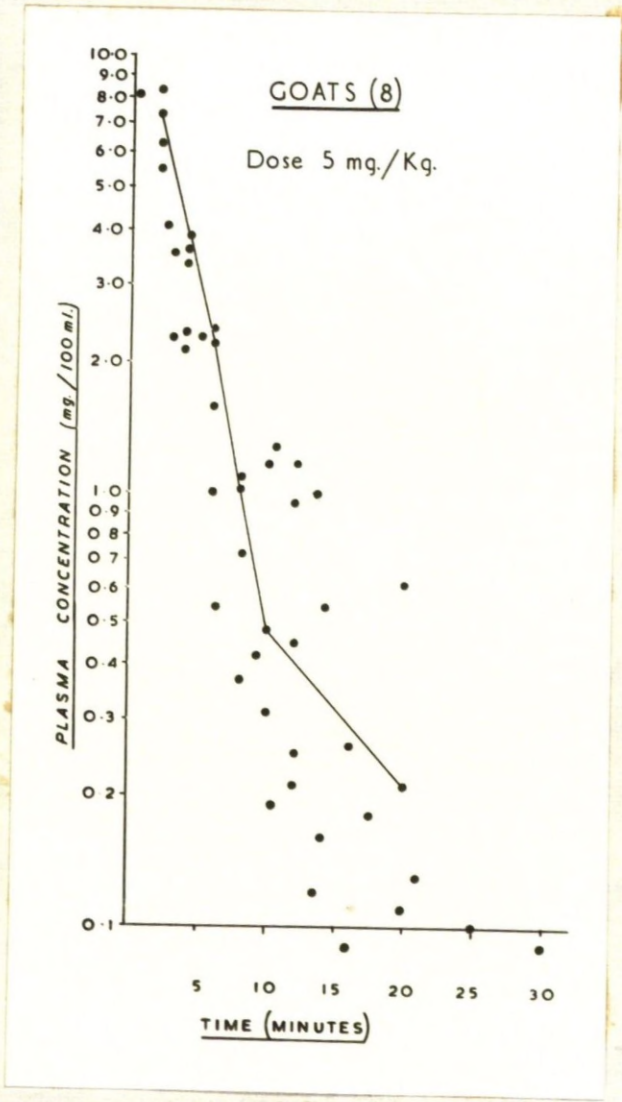
been in some cases rather inconclusive, due to the small number of animals investigated. In some of these animals the time period of estimation has only been for 20 or 30 minutes. The tests on these animals also had to be carried out rather blindly, since no reference has been found in the literature to a bromsulphthalein disappearance curve in the majority of the animal species investigated.

Figure 63 shows the disappearance graph which has been obtained in four cows given a dose of 5 mg./ Kg. body weight of bromsulphthalein. The observed concentrations obtained in one cow have been joined together and they appear to fall on a straight line. The observed concentrations in the other cows are in close proximity to the joined observed concentrations and therefore they have been depicted as individual points. Blood samples were only taken for a period of twenty minutes but, even so, by this time the plasma concentrations had fallen to low levels.

The result of a series of estimations on eight goats is depicted graphically in Figure 64. The logarithm plasma concentration is again plotted against time and shows 1 goat in which the observed plasma concentrations have been joined together,

Figure 64

The observed plasma concentrations in eight goats who were given a 5 mg./Kg. dose of bromsulphthalein.
The logarithm plasma concentration has been plotted against time over a period of 20 - 30 minutes.


GOATS (8)
Dose 5 mg./Kg.
PLASMA CONCENTRATION (mg./100 ml.)
10·0
9·0
8·0
7·0
6·0
5·0
4·0
3·0
2·0
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
5
10
15
20
25
30
TIME (MINUTES)

but all the other observed concentrations have been given as individual points. There is a suggestion in goats (similar to cats) that there may possibly be a bend or curve when the lower concentrations of dye are reached, but once again the interpretation is difficult because bromsulphthalein is removed rapidly from the circulation in these animals. It is not possible to draw any definite conclusions in either cats, goats or cows as to the type of disappearance curve, although the graphical results obtained do not yield the typical, double, exponential curve seen in dogs.

A slightly larger series of investigations were carried out on sheep and they consisted essentially of two groups (i) normal and (ii) abnormal.
Ten normal sheep were given a 5 mg./Kg. dose of bromsulphthalein and in the starved state, i.e. they were investigated in the morning after they had been kept indoors without food from the previous evening.
The results in the normal sheep are shown graphically in Figure 65, where the logarithm plasma concentration is plotted against time. Shown also in this Figure are the results obtained at a later date in five of these sheep. During the time interval, these animals

Figure 65

This shows the results of the observed plasma concentrations in ten normal sheep. Also shown are the observed plasma concentrations obtained 3 - 5 months later in five of these sheep who had a "fatty liver" meanwhile induced.
A dividing line can be placed between the 'normal' and 'abnormal' sheep.

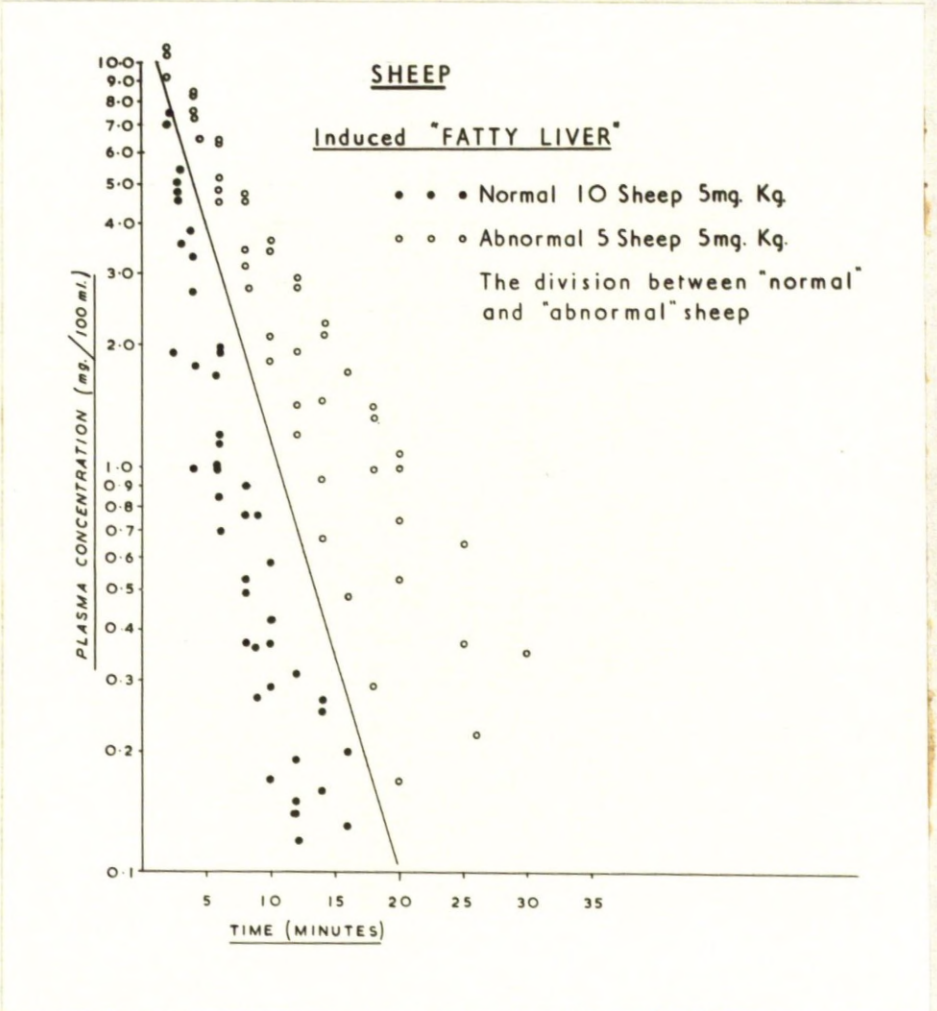
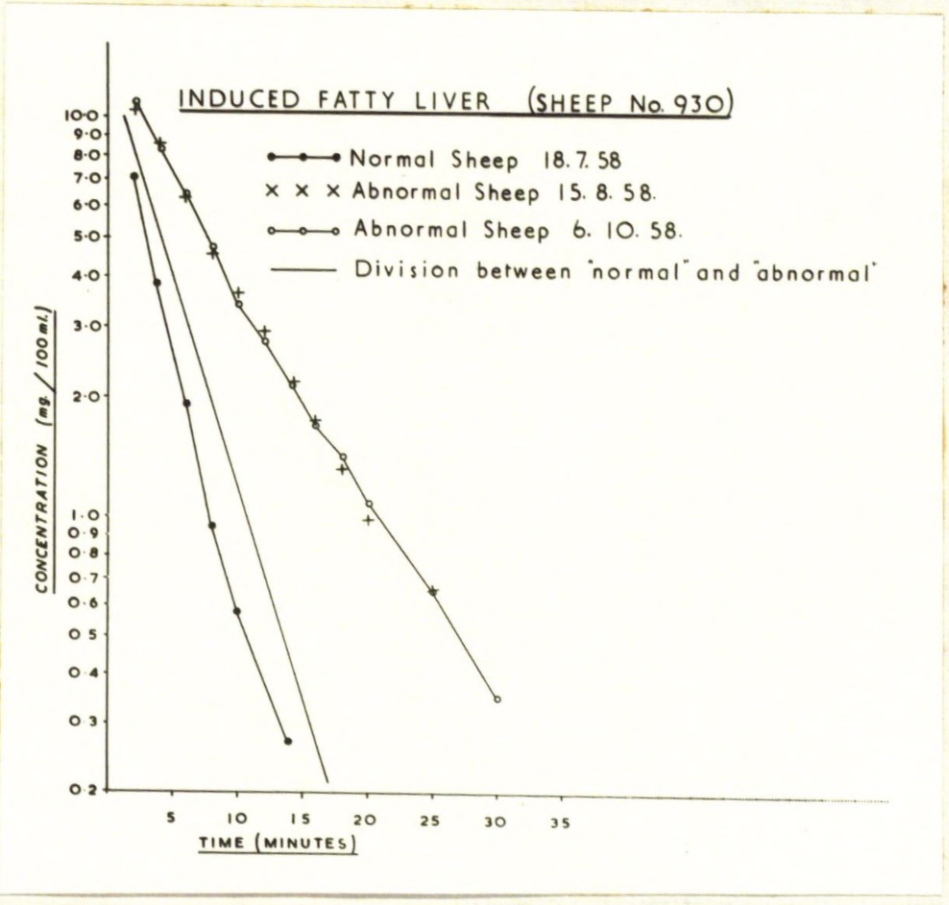

SHEEP
Induced "FATTY LIVER"
• • • Normal 10 Sheep 5mg. Kg.
o o o Abnormal 5 Sheep 5mg. Kg.
The division between "normal" and "abnormal" sheep
PLASMA CONCENTRATION (mg./100 ml.)
10·0
9·0
8·0
7·0
6·0
5·0
4·0
3·0
2·0
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
5
10
15
20
25
30
35
TIME (MINUTES)

Figure 66

This shows the result in sheep No. 930. The normal graph taken is compared with the observed plasma concentrations on the same animal after it had an induced "fatty liver". The dividing line between the normal and abnormal results in Figure 65 is also depicted. The logarithm plasma concentration is plotted against time.


INDUCED FATTY LIVER (SHEEP No. 930)
Normal Sheep 18. 7. 58
× × × Abnormal Sheep 15. 8. 58.
Abnormal Sheep 6. 10. 58.
Division between "normal" and "abnormal"
CONCENTRATION (mg / 100 ml.)
10·0
9·0
8·0
7·0
6·0
5·0
4·0
3·0
2·0
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
5
10
15
20
25
30
35
TIME (MINUTES)

had a "fatty liver" induced by dietetic methods. Some of the observations were carried out three months after the normal curves and some five months after the normal curves. It can be seen that a line can be drawn between the normal group and the abnormal group of sheep. Again, the impression is that a straight line would represent most of the joined points in the normal and abnormal sheep. Figure 66 shows the effect in one of these sheep (No. 930) which had three separate investigations. The normal curve for sheep No. 930 was taken on the 18th of July, 1958 and approximately one month later a repeat bromsulphthalein test was obtained, whilst a series of goats were being examined. Sheep No. 930 was one of the sheep in which a "fatty liver" was being induced and it was thought that it would be helpful to know whether or not any effect had been produced at this stage. It can be seen from the plotted, observed concentrations, that the level of plasma concentration is higher at any given time than noted in the normal state. The observed concentrations joined together were taken approximately three months after the initial control curve and correspond closely to those noted one month after the induced liver damage.

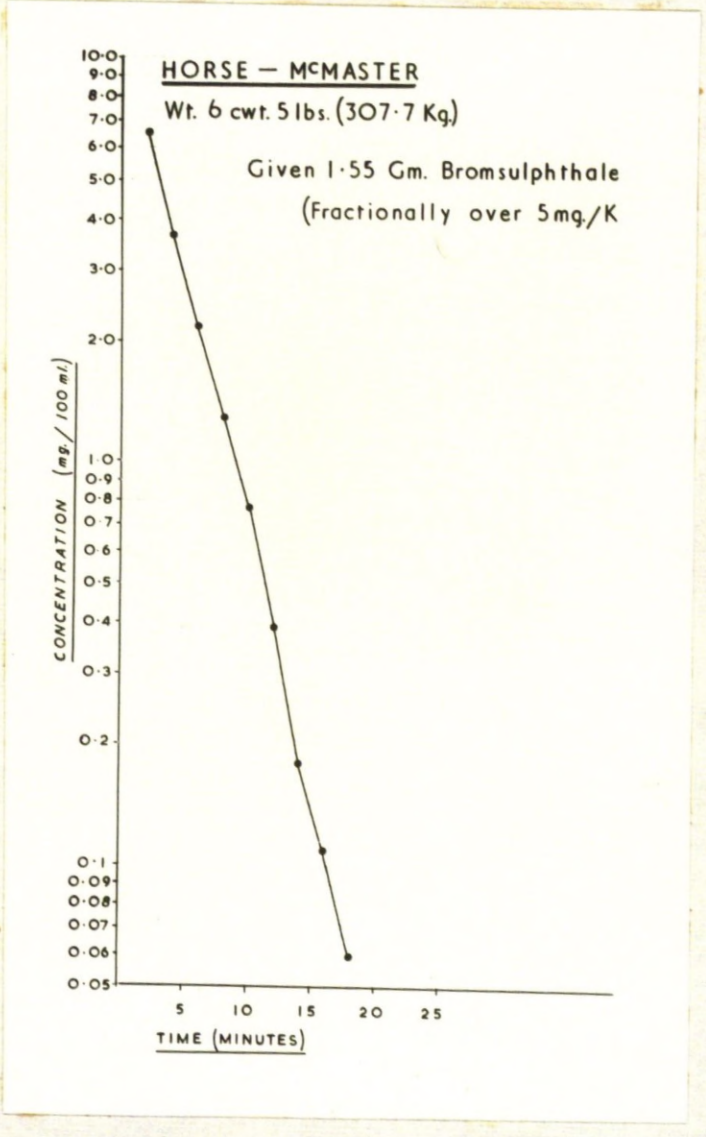
This Figure also shows the straight line division between "normal" and "abnormal".

It appears that in sheep (i) the observed plasma concentrations fall on a straight line when the logarithm plasma concentration is plotted against time, and (ii) that an induced "fatty liver" gives an assumed abnormal disappearance curve for sheep, following a 5 mg./Kg. dose of bromsulphthalein.

Whilst I was studying bromsulphthalein plasma disappearance in these various animal species at the Liverpool University Veterinary Field Station, I was informed that a horse, named McMaster, was suffering from cirrhosis of the liver and it was due to be killed the following day. It was suggested that it might be worth while carrying out a bromsulphthalein disappearance curve on this animal, and confirming the result with the post-mortem findings. Whilst realising that I had no idea of the normal disappearance curve of a horse, it was decided to carry out a controlled disappearance curve. Following the injection of a fraction over 5 mg./Kg. dose of bromsulphthalein, the observed concentrations were noted for a period of 18 minutes. It can be seen in Figure 67 that the dye disappeared rapidly from the

Figure 67

The plasma disappearance curve in a horse who was thought to be suffering from cirrhosis of the liver.
The post-mortem findings indicated no liver damage, confirming the impression that this is likely to be a perfectly normal disappearance curve for a horse.


HORSE — McMASTER
Wt. 6 cwt. 5 lbs. (307·7 Kg.)
Given 1·55 Gm. Bromsulphthale
(Fractionally over 5mg./K
CONCENTRATION (mg./100 ml.)
10·0
9·0
8·0
7·0
6·0
5·0
4·0
3·0
2·0
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0·09
0·08
0·07
0·06
0·05
5
10
15
20
25
TIME (MINUTES)

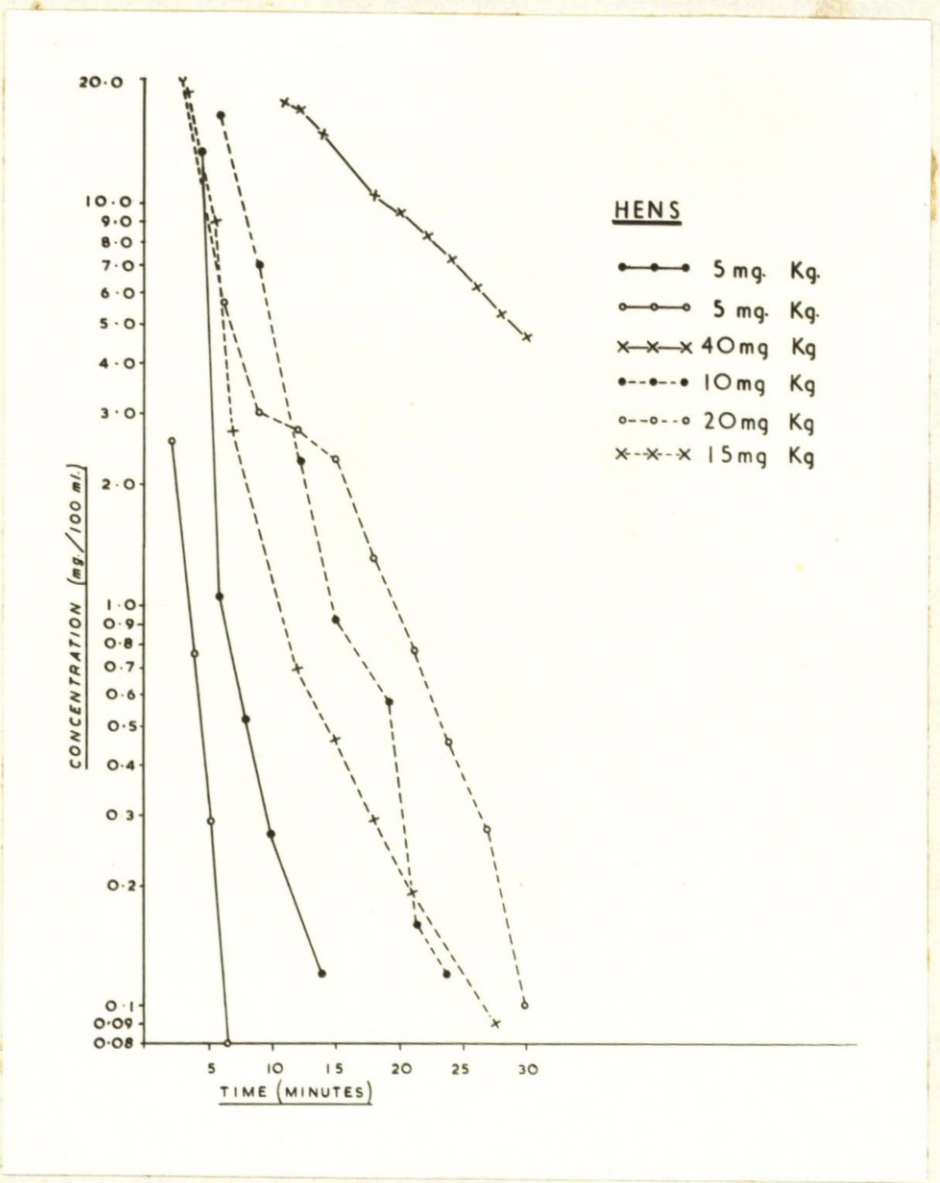
circulation with the impression of a straight line. The result seemed to indicate a perfectly normal liver function since the disappearance of dye was so rapid that it was considered unlikely that the liver of McMaster could be seriously affected. At post-mortem examination the following day, there was found to be no abnormality of the liver at all and no definite cause for the animal's illness was found.

It is interesting to note that the amount of bromsulphthalein given, over 1½ gr. means that in the horse the test is expensive, but, since the horse is a valuable and expensive animal, then a bromsulphthalein plasma disappearance test may help in the diagnosis, particularly in the type of horse which is likely to suffer from cirrhosis of the liver. The price of the bromsulphthalein test is minimal compared with the cost of a horse.

A series of experiments was carried out on hens, who were not anaesthetised, and at this point it is worth mentioning that out of all the animal species investigated, only rats, cats and dogs were anaesthetised. The hens had a main-wing vein catheterised under local anaesthesia. The disappearance of bromsulphthalein from the fowl is very rapid, as can be

Figure 68

The observed plasma concentrations in hens who were given varying doses of bromsulphthalein. Indicated in the graph are the results and the quantity of bromsulphthalein given.
The logarithm plasma concentration is plotted against time.

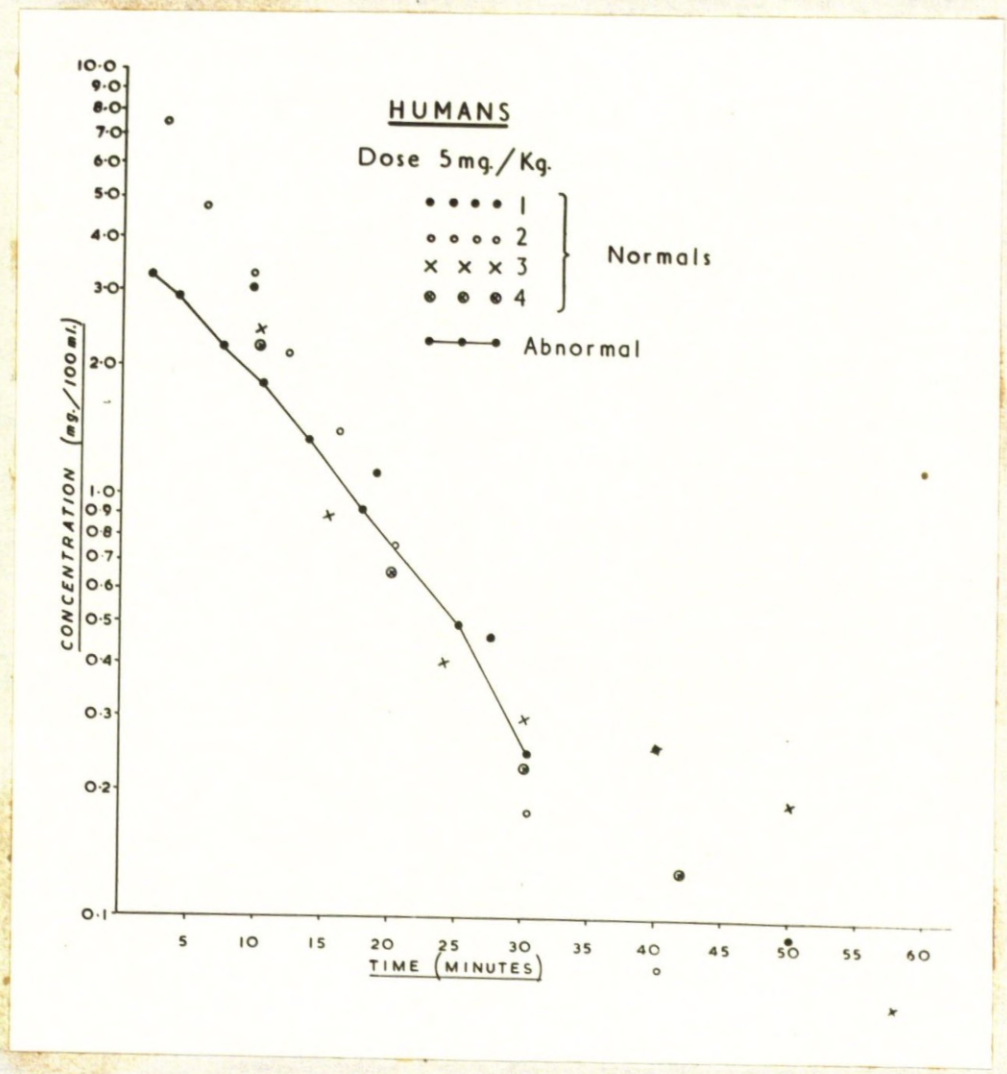

HENS
5 mg. Kg.
5 mg. Kg.
40 mg Kg
10 mg Kg
20 mg Kg
15 mg Kg
CONCENTRATION (mg/100 ml)
20·0
10·0
9·0
8·0
7·0
6·0
5·0
4·0
3·0
2·0
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0·09
0·08
5
10
15
20
25
30
TIME (MINUTES)

seen in Figure 68. Two hens given 5 mg./Kg. body weight would appear to get rid of the bromsulphthalein with great ease within 10 - 15 minutes. When larger doses are given, it can be seen that the observed concentrations tend to yield higher results at any given corresponding time. Some of the observed concentrations appear to be a little erratic and not fall on any straight or combination of straight lines. This was possibly due to the fact that in hens there was occasionally difficulty in obtaining reasonable samples of blood, and as the blood volume is small only small quantities could be taken but, compensating for this is the fact that the plasma volume is 70 - 80% of the blood volume in these birds. When one of the hens was given a large dose, 40 mg./Kg. body weight, the observed concentrations were relatively high and appeared to fall on a straight line. The observed period lasted only 30 minutes and it would have been more helpful had the experiment continued for a longer period of time.

A small series of experiments was carried out on human subjects. At the time, the candidate was working in the Physiology Department. The investigations on human volunteers were only just

Figure 69

The results obtained in four normal, human subjects, given a 5 mg./Kg. dose of bromsulphthalein. The abnormal curve depicted is that obtained from a man suffering from cirrhosis of the liver and who was given only 2 mg./Kg. body weight of bromsulphthalein.


HUMANS
Dose 5mg./Kg.
1
2
3
4
Normals
Abnormal
CONCENTRATION (mg./100ml.)
10·0
9·0
8·0
7·0
6·0
5·0
4·0
3·0
2·0
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
5
10
15
20
25
30
35
40
45
50
55
60
TIME (MINUTES)

commencing and these results were really preliminary experiments. Initially, there were a fair number of snags using the colorimetric method on the human plasma which had been used for all the other animal species. The main reasons being (i) If the subject had not fasted, there was an opalescence in the plasma, (ii) It was noted that whereas in all other species the plasma (if there was no haemolysis) appeared to be like water in appearance. This is not the case in the human subject where there was a definite yellow colour to be detected. The results which have been given in Figure 69 have been with the original method described already in the Section on Methods.

Subsequent work on human subjects which has been carried out in the Department of Physiology has, however, been with a modified colorimetric test. The principle behind it is similar to that given in the Methods here but instead of taking the readings for bromsulphthalein ahd haemoglobin, allowing for haemolysis, at the fixed filter wave-lengths of 580 and 425 , a third factor has to be taken into account, i.e. a certain amount of yellowness. The degree of yellowness for a particular individual also varies and therefore it has been necessary to take a

control sample in the human subject and from this, the factor for degree of yellowness, designated P, can be determined. It can then be subtracted or added to the necessary equations derived.

Returning to Figure 69, which shows four normal subjects in which 5 mg./Kg. of bromsulphthalein was given, it can be seen that these tend to fall on straight lines in all cases except one (the third curve appears to have a bend). On such a small series, however, it is difficult to be certain of the exact nature of affairs and a subsequent work by Goetzee, Richards and Thompson has shown that there is a double exponential curve in the majority of human subjects, but on occasions, the graphical and mathematical results yield only one straight, single exponential line. The abnormal result given here was obtained from a patient in a Medical Ward of the Royal Infirmary, under the care of Dr. C.A. Clarke, who had cirrhosis of the liver. This patient was being investigated prior to a porta-cavel shunt operation being carried out. The patient was only given a 2 mg./Kg. per body weight dose, but the observed concentrations can be seen to approximate to those obtained using a 5 mg./Kg. dose. Judging

from the dog experiments on the effect of the dose of bromsulphthalein, the smaller dose curve should be at a much lower level than the higher 5 mg./Kg. dose. Therefore, this was a distinctly abnormal result. The graphical results in this case again indicate a single straight line type of disappearance graph.

Discussion.

In many cases, the plasma disappearance bromsulphthalein curves have not been recorded (as far as I can ascertain from the literature) in many of the species which I have covered in this Section. Cats, generally speaking, have not been the animals in general used for analysis. Dogs have been studied by many workers and they are the most popular experimental animal for bromsulphthalein studies. Most workers who have followed the disappearance of bromsulphthalein have only been interested in the first portion of the graph, that is, the initial disappearance phase. The fact that a bend has occurred at the later time intervals has generally been ignored. There is no reference (as far as I can ascertain) to the disappearance curves in either cows, goats or sheep. In the case of the sheep examined in this series, it

would appear to yield valuable evidence of impaired liver function. Assuming more normal sheep could be investigated and the normal disappearance pattern ascertained, with more precision, then the bromsulphthalein disappearance curve would give valuable information regarding impairment of liver function. In the solitary horse examined, all that one can say is that there appeared to be no evidence of liver function and indeed, no evidence of liver damage was detected after pathological examination of the liver. The larger the animal, of course, the greater is the quantity of bromsulphthalein that needs to be given and it is a relatively expensive substance to use.

A reference to a disappearance curve in hens has been given by Campbell (1957). He, however, was determining the amount of liver damage by the difference in dye removal between 5 - 10 and 10 - 15 minutes blood samples, when the animals had been given a 20 mg./Kg. dose of bromsulphthalein. Bromsulphthalein is used as a test in humans, but the usual clinical test has been to estimate either one or two samples of blood at varying times between 30 and 60 minutes. McDonald (1939) suggested that serial plasma estimations would be useful in differentiating

various types of liver damage, but this has generally not found favour, possibly due to the fact that repeated venepuncture is often required in order to obtain serial blood samples.

There are several possible explanations for the different types of disappearance curves which have been obtained in the various animal species. If we accept a typical double exponential curve, as given in Figure 70, to be the normal curve, as it has been demonstrated in dogs, then if the initial phase is very short, as indicated in Figure 71, then there may only be one observed point at this early stage, or, in fact, there may be no observation during this time. Subsequent results in the example depicted graphically would appear to fall on the solid straight line. On the other hand, the second slower phase may be below the lower limit of colorimetric analysis. In the example depicted in Figure 72, for instance, the lower limit of accuracy is accepted as 0.1 mg. and the second slower phase which occurs after approximately fifteen minutes is below this limit and therefore the observed readings would not be satisfactory, being negative or not recordable. A further possibility is given in Figure 73, in which

Figure 70

This gives a typical, double, exponential curve, as has been found in dogs.

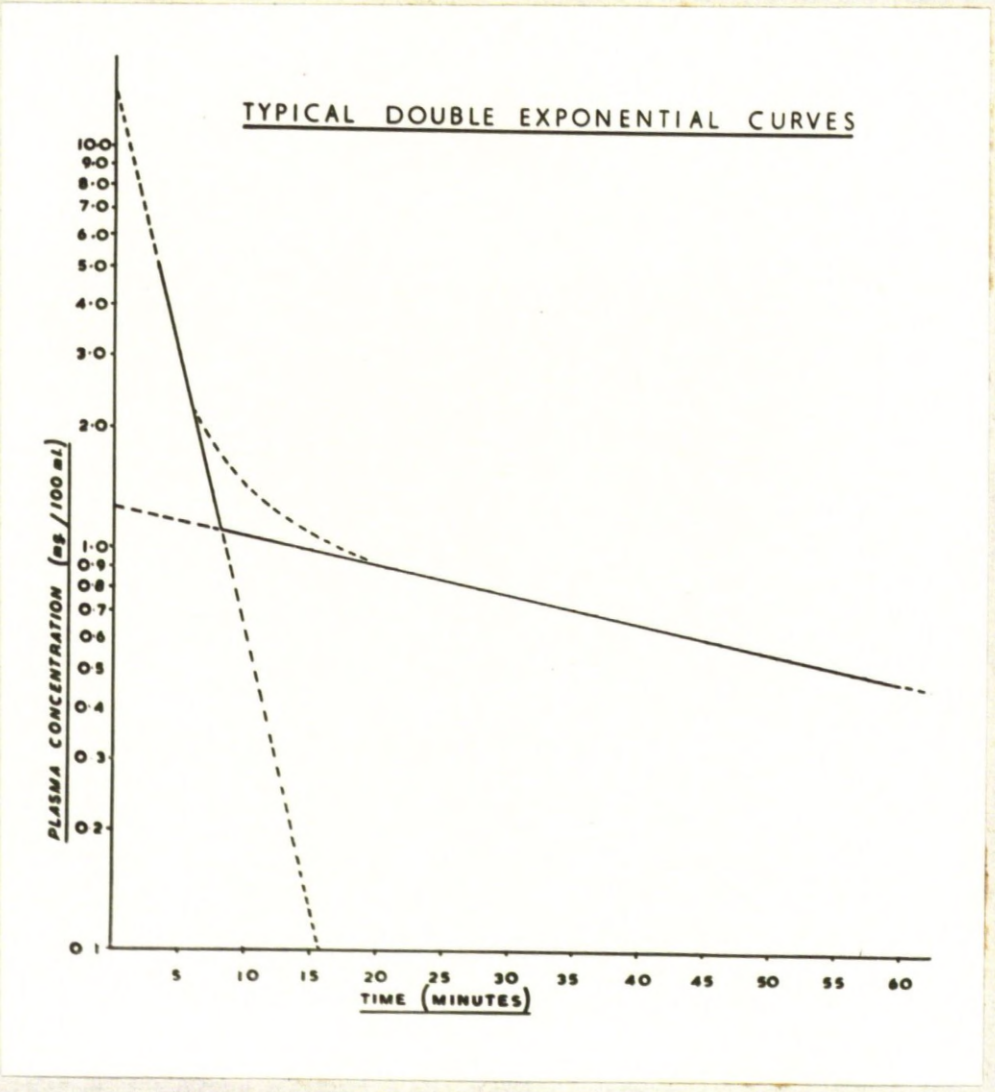

TYPICAL DOUBLE EXPONENTIAL CURVES
PLASMA CONCENTRATION (mg / 100 ml)
100
9·0
8·0
7·0
6·0
5·0
4·0
3·0
2·0
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
5
10
15
20
25
30
35
40
45
50
55
60
TIME (MINUTES)

Figure 71

This Figure indicates that if the double, exponential curve has a very short initial phase, then this is likely to be completely missed or, at the most, only one observation obtained and this may possibly be ignored.

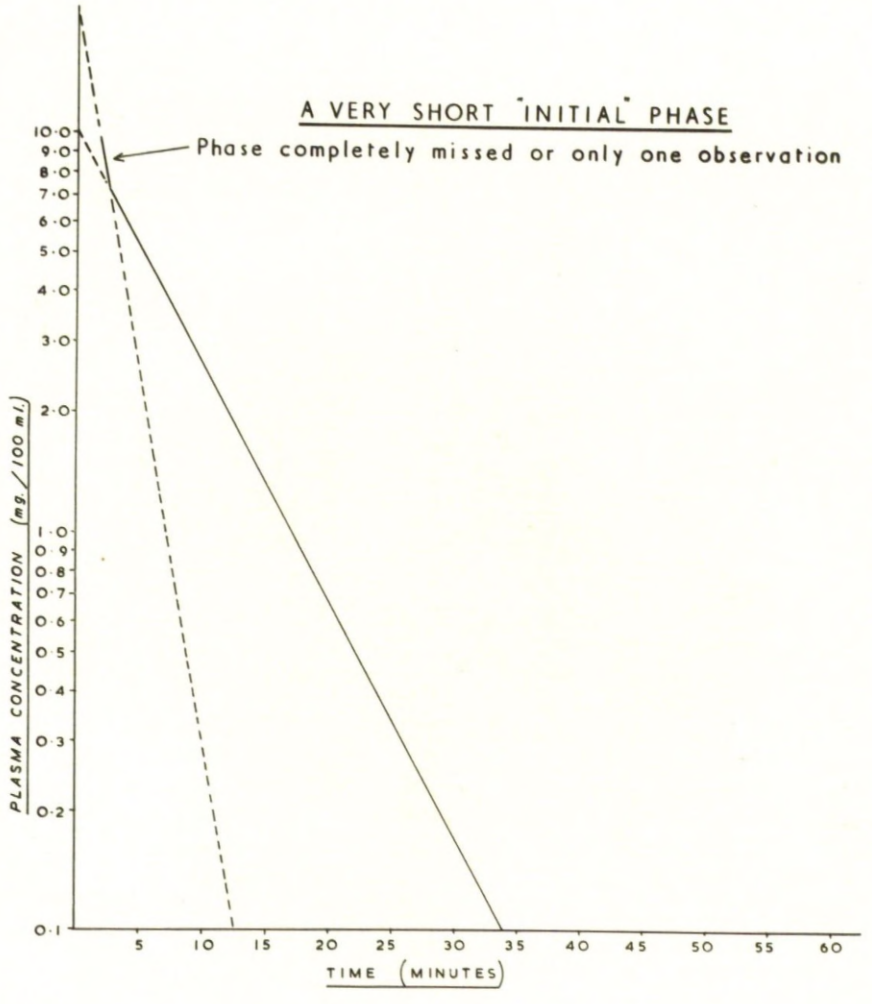

A VERY SHORT "INITIAL" PHASE
Phase completely missed or only one observation
PLASMA CONCENTRATION (mg./100 ml.)
10·0
9·0
8·0
7·0
6·0
5·0
4·0
3·0
2·0
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
5
10
15
20
25
30
35
40
45
50
55
60
TIME (MINUTES)

Figure 72

This shows a double, exponential curve in which the second or slower phase is below the lower limit of colorimetric estimation.

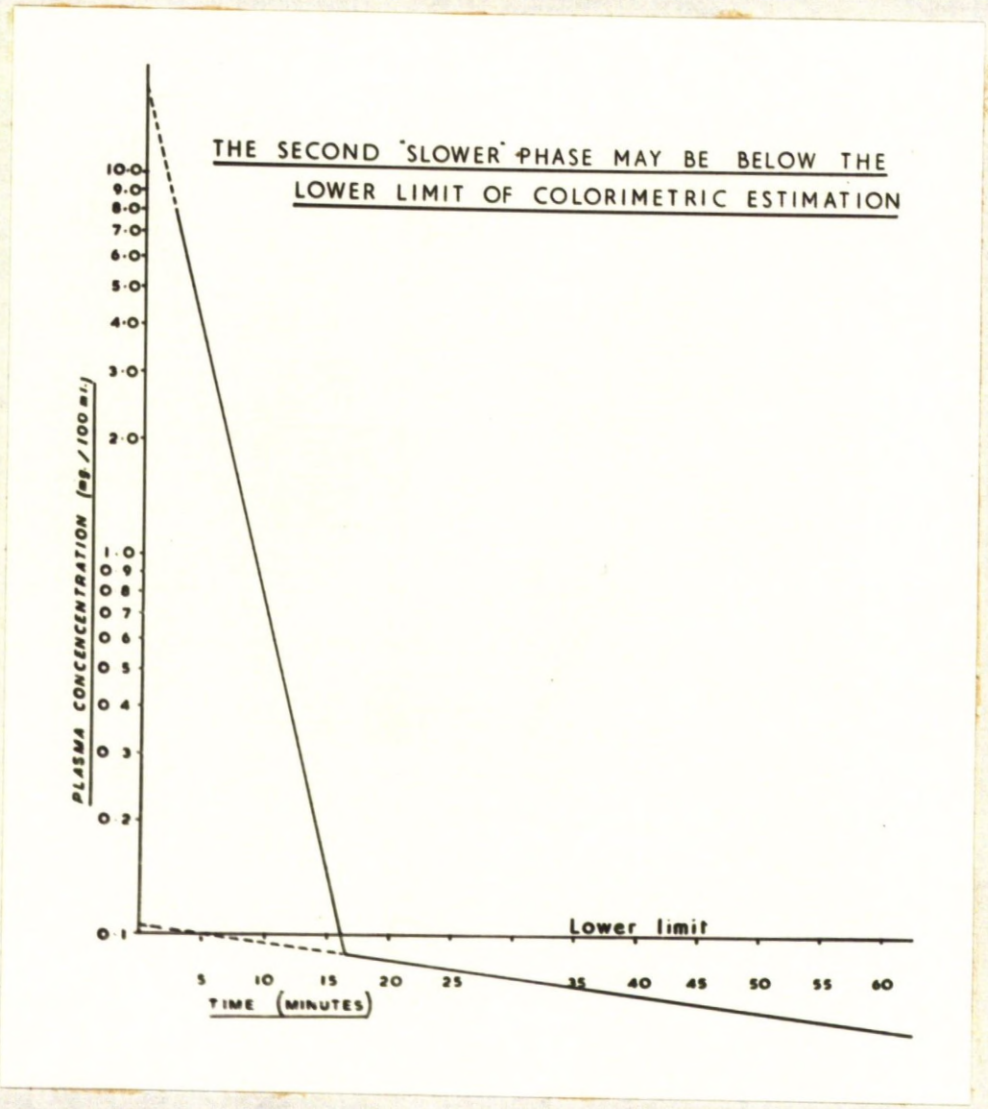
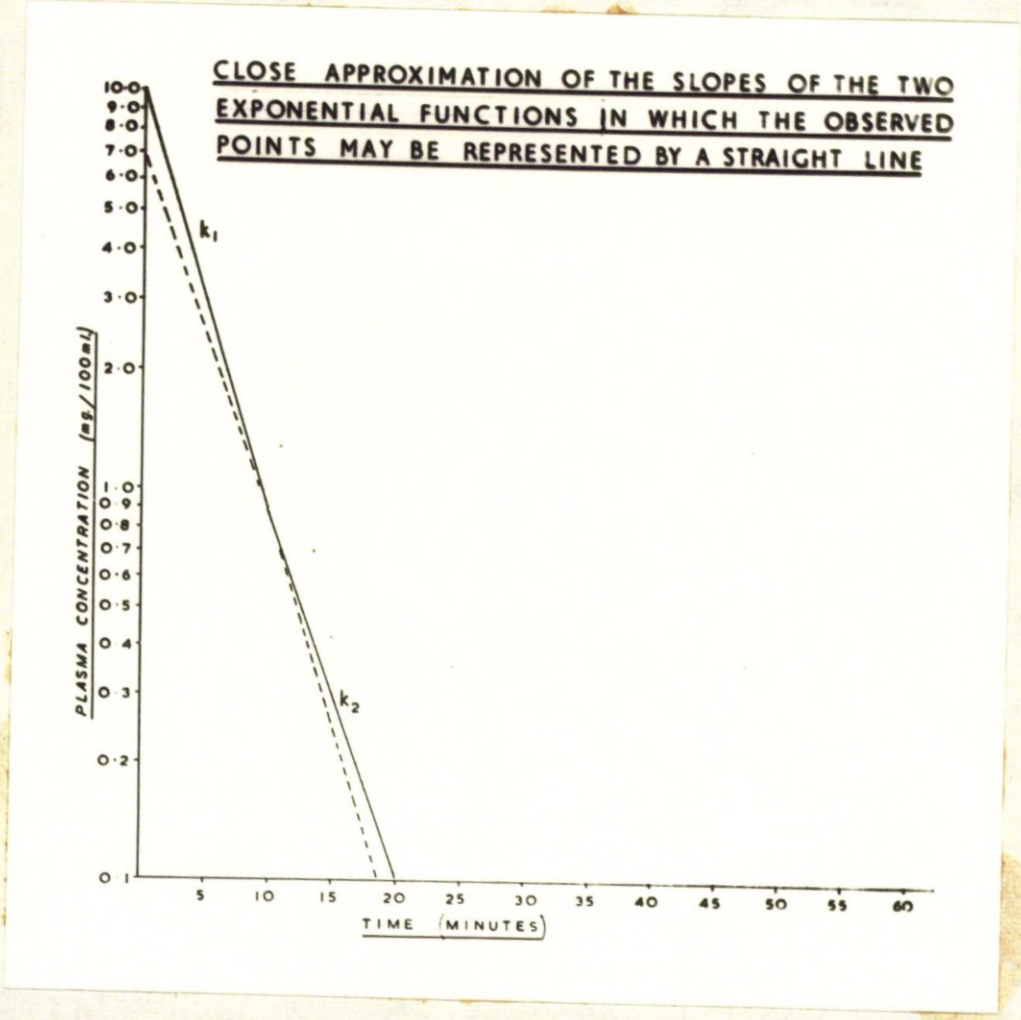

THE SECOND "SLOWER" PHASE MAY BE BELOW THE LOWER LIMIT OF COLORIMETRIC ESTIMATION
PLASMA CONCENTRATION (mg / 100 ml)
10·0
9·0
8·0
7·0
6·0
5·0
4·0
3·0
2·0
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
Lower limit
5
10
15
20
25
35
40
45
50
55
60
TIME (MINUTES)

Figure 73

A close approximation of the slopes of the two exponential functions in which the constants k_1 and k_2 are very similar and may, due to experimental error, be represented as a single, straight line when the observed logarithm plasma concentrations are plotted against time.


CLOSE APPROXIMATION OF THE SLOPES OF THE TWO EXPONENTIAL FUNCTIONS IN WHICH THE OBSERVED POINTS MAY BE REPRESENTED BY A STRAIGHT LINE
PLASMA CONCENTRATION (mg/100ml)
10·0
9·0
8·0
7·0
6·0
5·0
4·0
3·0
2·0
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
k_1
k_2
5
10
15
20
25
30
35
40
45
50
55
60
TIME (MINUTES)

there is a close approximation of two exponential functions. In other words, the slopes of the two lines k_1 and k_2 depicted in Figure 32, closely approximate one another. The observed concentrations with their inevitable errors might well appear to fall on a straight line (a combination of the two separate lines). This may be the explanation as to why some of the results presented to Mr. Young for analysis in normal intact animals were found to be unsuitable for the electronic computer, because it was not possible to distinguish with any accuracy between the slopes of k_1 and k_2 when they approximate to each other very closely.

There is another possibility, although this has not yet been investigated, to account for the difference in bromsulphthalein disappearance curve in the animals, apart from the above explanation. Since bromsulphthalein is bound to plasma albumin and to liver protein it is quite possible that the relative amounts of protein present in the blood and the liver determine the type of disappearance graph. The disappearance curve from the plasma reflects the proportion of protein within the blood compartment to the proportion of dye in the liver and the ability of

the liver to get rid of the dye into the bile.
In the different species examined, the plasma volume and liver volume (or weight) ratio will vary, as will the ratio of the protein content in each of these sites.

E. THE EFFECT OF VARIOUS SUBSTANCES ON THE BROMSULPHTHALEIN DISAPPEARANCE CURVE

The Effect of Bilirubin

Figure 74 shows the effect of bilirubin on the bromsulphthalein disappearance curve. This Figure shows the normal control curve and a disappearance curve which was taken one week later, when bilirubin was injected 18.25 minutes after the bromsulphthalein had been given. It can be seen that there is a close approximation between the initial part of the curve, prior to bilirubin being given. Following bilirubin, the plasma concentrations appeared to be elevated slightly but there was no real difference in the subsequent pattern of bromsulphthalein disappearance. If anything, the subsequent rate of removal of bromsulphthalein seems to be a little more rapid. The effect of biligrafin when given 20 minutes before the standard 5 mg./Kg. dose of bromsulphthalein is also shown in this Figure. It can be seen that the observed concentrations in this case are at all times higher than the normal control curve. The curve obtained is still within the normal accepted limits, although compared with its own control, it is elevated.

Figure 75 shows the control curve of a dog given 5 mg./Kg. body weight. Six weeks later the

Figure 74

The effect of bilirubin. The control bromsulphthalein disappearance curve is compared with the curves obtained after bilirubin had been given 20 minutes prior to bromsulphthalein, and also when bilirubin is given 18.25 minutes after the bromsulphthalein injection alone, at zero time. In each case 5 mg./Kg. body weight of bromsulphthalein has been given and a dose of 1 mg./Kg. body weight of bilirubin has been given.

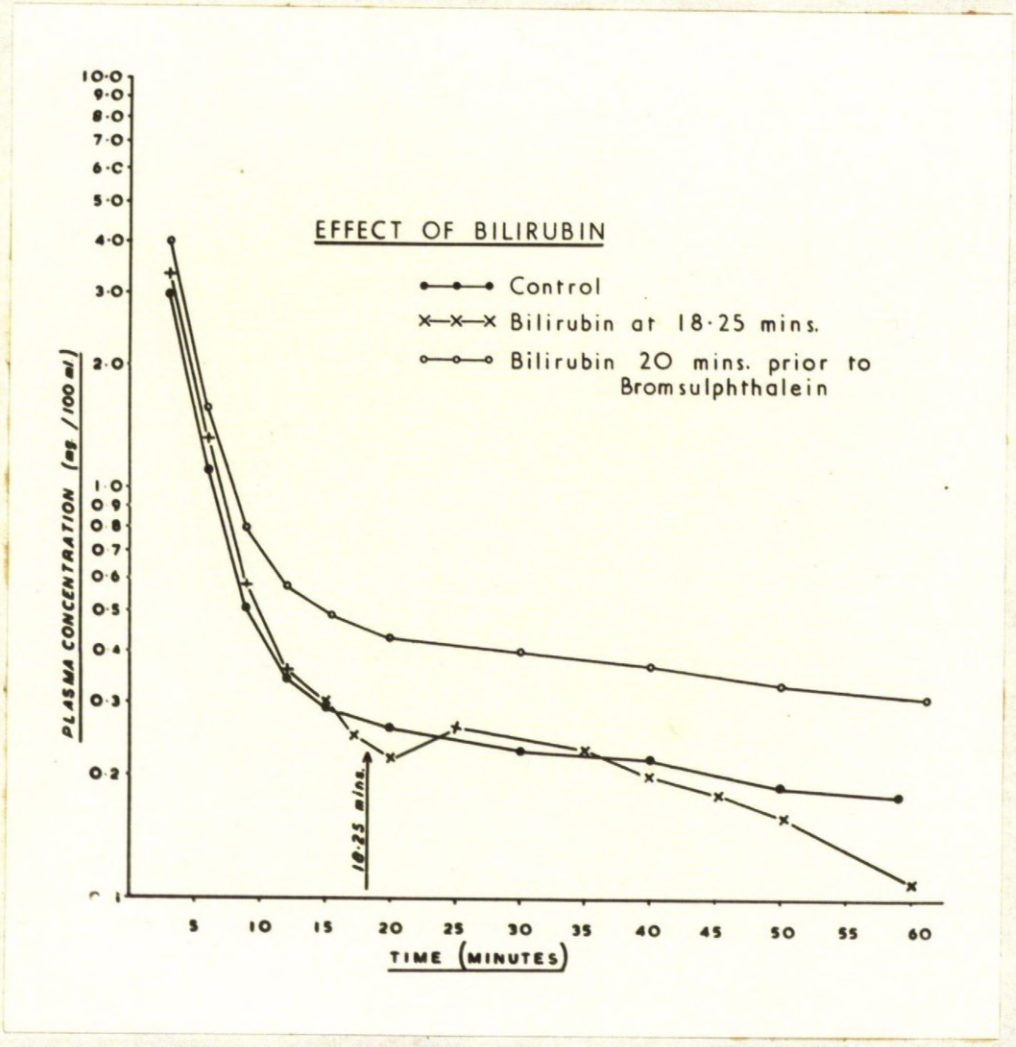
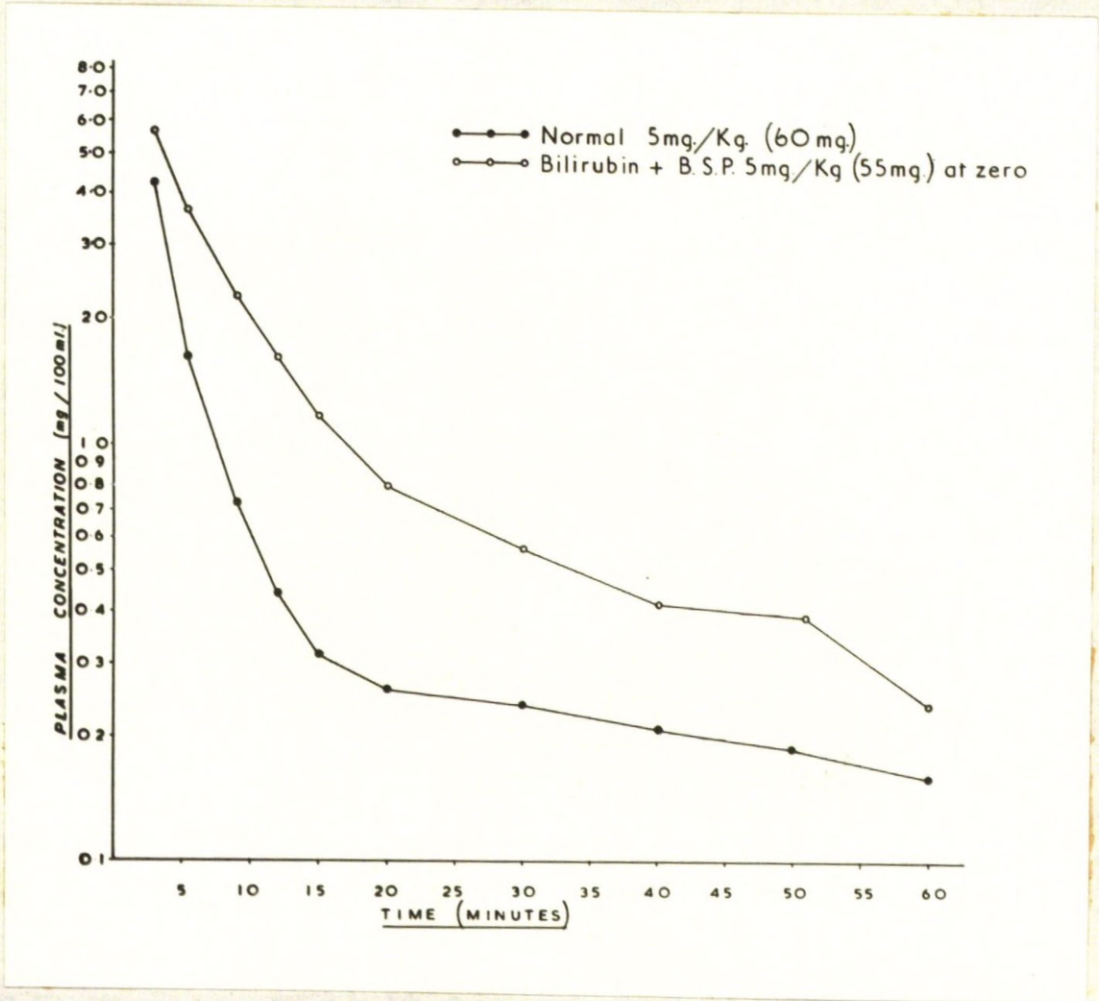

EFFECT OF BILIRUBIN
Control
Bilirubin at 18·25 mins.
Bilirubin 20 mins. prior to Bromsulphthalein
PLASMA CONCENTRATION (mg / 100 ml)
10·0
9·0
8·0
7·0
6·0
5·0
4·0
3·0
2·0
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
18·25 mins.
5
10
15
20
25
30
35
40
45
50
55
60
TIME (MINUTES)

Figure 75

This shows the effect of bromsulphthalein and bilirubin given together at zero time compared with the normal disappearance curve. In each case, a standard 5 mg./Kg. dose of bromsulphthalein has been given, but when the second disappearance curve was taken, the animal weighed 1 Kg. less than at the time of the normal, disappearance curve. The dose of bilirubin was 1 mg./Kg. body weight.

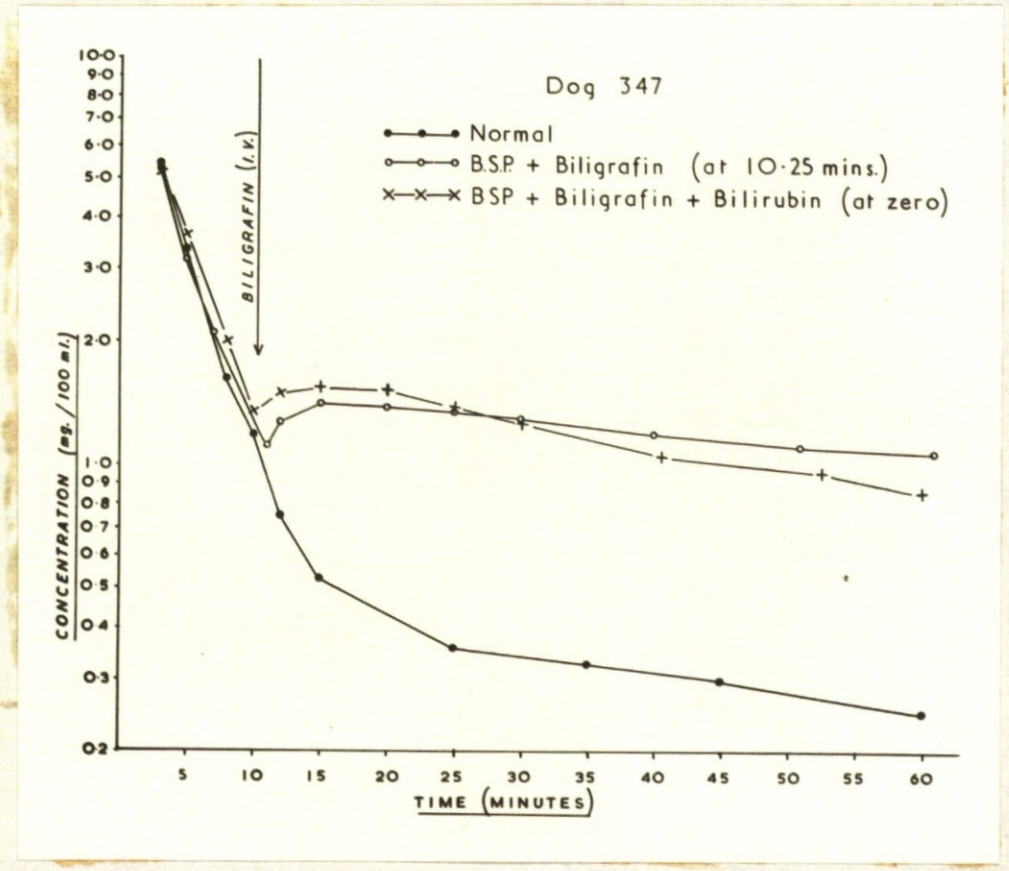

Normal 5mg./Kg. (60 mg.)
Bilirubin + B.S.P. 5mg./Kg (55mg.) at zero
PLASMA CONCENTRATION (mg./100ml.)
8·0
7·0
6·0
5·0
4·0
3·0
2·0
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
5
10
15
20
25
30
35
40
45
50
55
60
TIME (MINUTES)

animal had lost 1 Kg. in weight and the dose of bromsulphthalein (5 mg./Kg.) was only 55 instead of the 60 mg. given previously. On the second occasion, bilirubin (1 mg./Kg.) and bromsulphthalein were given together. It can be seen that the plasma concentrations on the second occasion are much higher than the control observation at any given time. The observed concentrations when bilirubin and bromsulphthalein are given simultaneously are at the upper limits of the expected normal for 'intact' dogs. The observed concentrations which appear on the "bend" appear to be just outside the normal limits.

Figure 76 shows the results of experiments with bilirubin in the case of dog 347. This animal was also used for the biligrafin series. A control disappearance curve was obtained and some time later the effect of Biligrafin Forte (1 mg./Kg.) given 10.25 minutes after the standard dose of bromsulphthalein was noted. Following the injection of biligrafin, the plasma concentration is elevated and the disappearance of bromsulphthalein from the plasma is delayed. The graphical results would suggest that the slopes of the line subsequent to the biligrafin injection correspond reasonably well with the slope of

Figure 76

The experiments on dog 347. In this animal a normal disappearance curve is given and the effect of biligrafin given intravenously at 10.25 minutes is determined on two separate occasions, (i) when bromsulphthalein is given alone and (ii) when bromsulphthalein and bilirubin are given simultaneously at zero time.


Dog 347
Normal
B.S.P. + Biligrafin (at 10-25 mins.)
BSP + Biligrafin + Bilirubin (at zero)
BILIGRAFIN (I.V.)
CONCENTRATION (mg./100 ml.)
10·0
9·0
8·0
7·0
6·0
5·0
4·0
3·0
2·0
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
5
10
15
20
25
30
35
40
45
50
55
60
TIME (MINUTES)

the second slower phase of the control curve. The effect when biligrafin (1 mg./Kg.) is given intravenously at the same time (10.25 minutes) on the disappearance curve when the 5mg./Kg. dose of bromsulphthalein is given simultaneously with an intravenous injection of bilirubin is also shown. It can be seen that the effects on this occasion are similar to those previously given with biligrafin and bromsulphthalein alone. The only difference detected is that the slight elevation of bromsulphthalein in the plasma is slightly delayed when bilirubin is given with bromsulphthalein. There is also the impression (unfortunately not confirmed by the collection of bile) that the subsequent removal of bromsulphthalein from the plasma may be delayed, even more than when bromsulphthalein had been given alone.

Summary

In these three experiments which have been depicted graphically, a standard dose of bilirubin was given (1 mg./Kg.). In the first instance (Figure 74) bilirubin was given after the majority of the bromsulphthalein had disappeared from the plasma, and did not appear to affect the normal disappearance of bromsulphthalein. When, however,

given simultaneously with bromsulphthalein, there appeared to be some delay in the removal of bromsulphthalein from the plasma, although the resulting plasma concentrations were within the accepted normal for 'intact dogs'. The effect of giving bilirubin and bromsulphthalein simultaneously is even more marked (Figure 75). Although a standard dose of bromsulphthalein was given in this dog, the animal had lost 1 Kg. in weight between the first and the second experiment and therefore the results may not be strictly comparable. The impression is that bilirubin in the quantity given slightly interfered with the normal disappearance pattern of bromsulphthalein when the two dyes were injected either together, or bilirubin given before bromsulphthalein. The final experiment, depicted in Figure 76, confirms the impression gained from Figure 74 that when bilirubin is given after bromsulphthalein it does not apparently interfere with the normal excretion pattern of bromsulphthalein, even when challenged with another substance (Biligrafin). In these experiments, bilirubin appears to behave differently from that noted by Cantarow, Wirts, Snape and Miller (1948) who found that when bilirubin and bromsulph-

thalein were given together, bromsulphthalein was excreted as if it had been given alone. Their conclusions were based on the bile excretion of bromsulphthalein (not confirmed in the experiments given above) and since the doses of bilirubin are different, the results cannot strictly be compared.

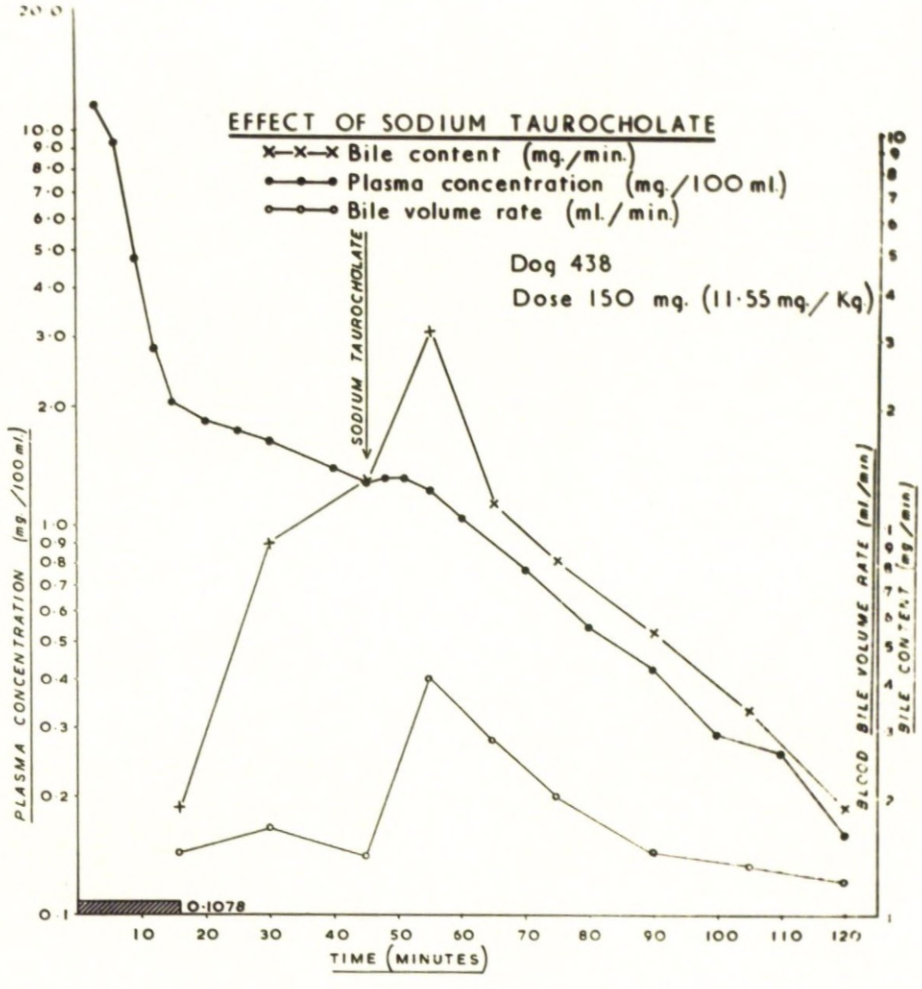
The effect of Sodium Taurocholate

A single experiment in which Sodium Taurocholate was given to dog 438 is detailed in the next three Figures. This dog had an acute biliary fistula operation performed and the dose of bromsulphthalein given exceeded the standard dose. The animal was given 150 mg. (i.e. 11.55 mg./Kg. body weight) intravenously after a control period of 45 minutes. Forty-five minutes after bromsulphthalein, 250 mg. of Sodium Taurocholate was given intravenously. Some of the effects of Sodium Taurocholate are depicted in Figure 77, where the logarithm of the observed plasma concentration is plotted against time. Once again, there is an initial rapid phase, followed by a second slower phase. After Sodium Taurocholate was given at forty-five minutes, the subsequent plasma concentrations appear to be elevated for a short period of time and then the bromsulphthalein disappears at a slightly increased rate compared with the "expected" slope of the second slower phase of the pre-Sodium Taurocholate phase of the bromsulphthalein disappearance graph. The logarithm bile volume rate (mls./min.) is also shown in this Figure plotted

Figure 77

The effect of Sodium Taurocholate.

The logarithm plasma concentration is plotted against time and the typical double exponential type of curve is shown, an initial rapid phase, followed by the second slower phase. Sodium Taurocholate was given at 45 minutes after bromsulphthalein and there appears to be a slight increase in the plasma concentration for a short time, after which the dye disappears relatively quickly from the plasma. Also shown are the logarithm of the bile volume rate (mls./min.) and the logarithm bile content(mg./min.).
In the left-hand side bottom corner, the average control bile volume rate (mls./min.) is given, it being 0.1078 mls./min. during the control period of 45 minutes prior to the injection of bromsulphthalein. This dog was given a dose of 150 mg. (11.55 mg./Kg.).


EFFECT OF SODIUM TAUROCHOLATE
Bile content (mg./min.)
Plasma concentration (mg./100 ml.)
Bile volume rate (ml./min.)
Dog 438
Dose 150 mg. (11·55 mg./Kg.)
SODIUM TAUROCHOLATE
PLASMA CONCENTRATION (mg./100 ml.)
BLOOD BILE VOLUME RATE (ml./min.)
BILE CONTENT (mg./min.)
0·1078
TIME (MINUTES)

against time. The "hatched-out" portion in the lower left hand side of the graph shows the normal bile volume rate during the 45 minutes control period. The control bile volume was 0.1078 mls./min. The bile volume rate increases with the injection of bromsulphthalein and after Sodium Taurocholate there seems to be an increased effect but there is no control value with which to compare it. The subsequent bile volume rate falls rather rapidly, so that the bile volume rate at 120 minutes is approximately equal to the rate noted 15 minutes after bromsulphthalein. The maximum bile volume rate was just over 3.1 mls. per minute, and occurred approximately ten minutes after the injection of Sodium Taurocholate. In this graph the bile content of bromsulphthalein in mg. per minute is shown. During the three 15 minute periods following bromsulphthalein the bile content was (i) 0 - 15 minutes, it was 0.143 mg, per minute, (ii) 15 - 30 minutes it was 0.165 mls. per minute and (iii) between 30 - 45 minutes the bile content was 0.140 mg. per minute. Following the injection of Sodium Taurocholate (a known choleretic substance) the bile volume content of bromsulphthalein rapidly increased to just over 0.4 mg. per minute. The subsequent 10 minute

period observations revealed a gradual fall in the rate of bromsulphthalein excretion but for a period of 45 minutes, after Sodium Taurocholate had been given, the average rate of production was above the rate obtained at 45 minutes (0.140 mg./min.)

The effect of Sodium Taurocholate on bile volume in millilitres per minute in dog 438 and on the bromsulphthalein excretion in milligrammes per minute, is depicted separately in Figure 78. During the 45 minute control period the bile volume rate seemed reasonably constant. Following bromsulphthalein there was a 30 - 40% increase in the bile volume rate and when Sodium Taurocholate was given at 45 minutes, after a short time interval the bile volume rate increased to a maximum of almost 4 times the control volume rate and the choleretic effect was maintained for a period of 30 minutes, but at a reduced rate. The bile volume rate was higher than the control volume rate for approximately 150 minutes after the bromsulphthalein injection, that is, 105 minutes after the Sodium Taurocholate injection. The bromsulphthalein content in bile shown in this Figure reveals a rapid increase in the amount of dye in mg. per minute, prior to taurocholate being given,

Figure 78

The effect of Sodium Taurocholate.

This shows a comparison of the bile volume (mls./min) and the bromsulphthalein excretion rate (mg./min.) during the control period, following the injection of bromsulphthalein and again following the intravenous injection of 250 mg. of Sodium Taurocholate.
Compared with the control there is an increase in the bile volume rate following bromsulphthalein with a marked, but relatively short increase, following the injection of Sodium Taurocholate. The peak excretion of bromsulphthalein corresponds with the choleretic effect of Sodium Taurocholate, but it also occurs during the expected time peak of dye excretion, following an intravenous dose of bromsulphthalein.

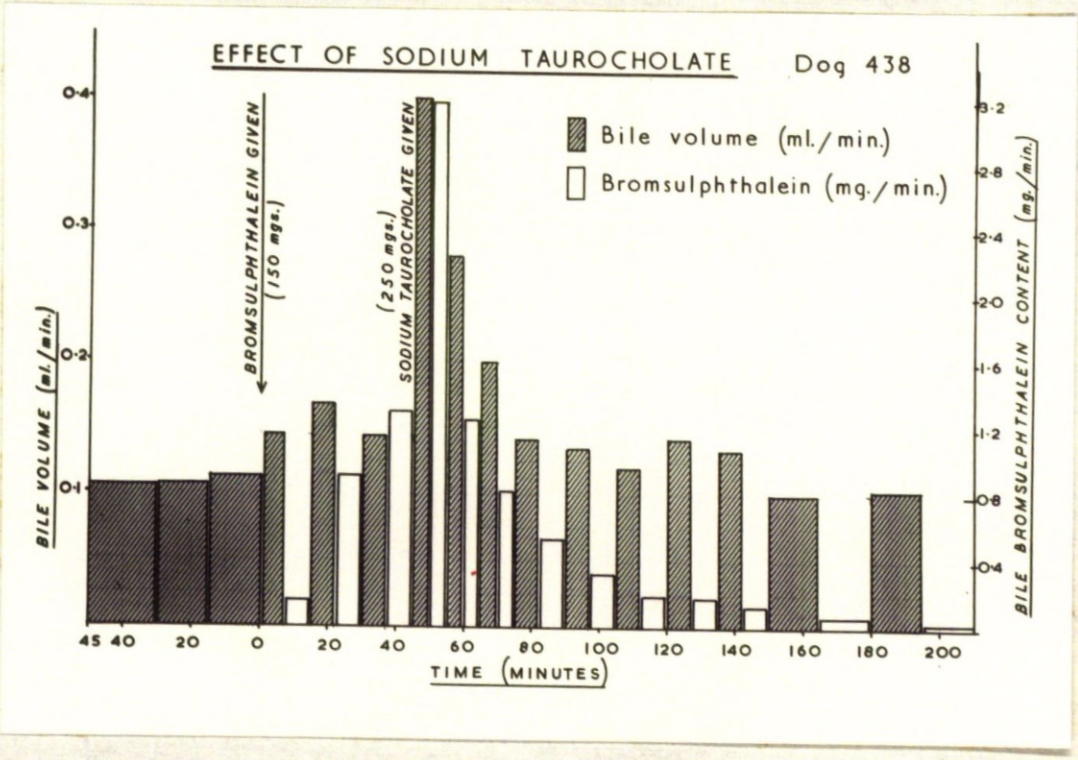
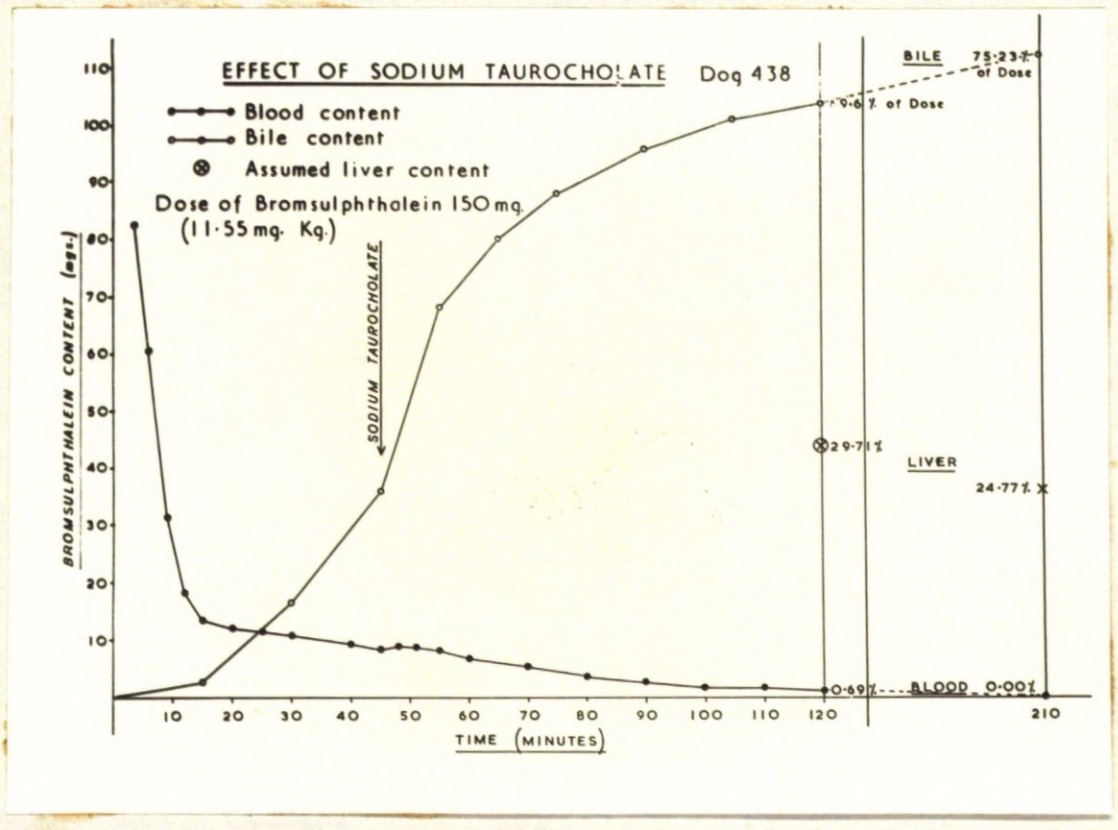

EFFECT OF SODIUM TAUROCHOLATE
Dog 438
Bile volume (ml./min.)
Bromsulphthalein (mg./min.)
BROMSULPHTHALEIN GIVEN (150 mgs.)
SODIUM TAUROCHOLATE GIVEN (250 mgs.)
BILE VOLUME (ml./min.)
BILE BROMSULPHTHALEIN CONTENT (mg./min.)
0·4
0·3
0·2
0·1
3·2
2·8
2·4
2·0
1·6
1·2
0·8
0·4
45 40 20 0 20 40 60 80 100 120 140 160 180 200
TIME (MINUTES)

Figure 79

The effect of Sodium Taurocholate.

The temporal distribution of the dye in the blood and bile is shown in dog 438, who had a dose of bromsulphthalein, 150 mg. (11.55 mg./Kg.) given at zero time. At 45 minutes, 250 mg. of Sodium Taurocholate was injected intravenously. The blood content of dye has been obtained assuming a plasma volume of 50 mg./Kg. body weight. At the end of 60 minutes, 75 mg. of dye has been recovered (50% of the dose), i.e. within the expected value for acute biliary fistula animals. At 120 minutes, 69.6% of the dose had been recovered and at the end of the experiment (210 mins.) 75.23% of the dose had been recovered in the bile. The blood content shows a slight increase in the actual amount of bromsulphthalein present in the blood after Sodium Taurocholate. The assumed liver content of dye, based on the assumption that the liver contains any dye which is not present in the blood or bile, gives a value at 120 and 210 minutes respectively of 29.71% and 24.77%.


EFFECT OF SODIUM TAUROCHOLATE Dog 438
Blood content
Bile content
Assumed liver content
Dose of Bromsulphthalein 150 mg.
(11·55 mg. Kg.)
SODIUM TAUROCHOLATE
BROMSULPHTHALEIN CONTENT (mgs.)
TIME (MINUTES)
BILE 75·23% of Dose
9·6 % of Dose
29·71%
LIVER
24·77%
0·69%
BLOOD 0·00%

after which it gradually falls.

Using an assumed plasma volume rate of 50 mls. per Kg. body weight, the blood content of dye is depicted in Figure 79 and it is compared with the recovery rate of bromsulphthalein in the bile. The distribution pattern tends to follow the distribution predicted by the mathematical model, with the exception that there is a slightly elevated blood content of dye after Sodium Taurocholate has been injected, but the percentage of dye recovered in the bile at the end of 60 minutes is approximately 50%, which would appear to be very reasonable. The blood content of bromsulphthalein at the end of 120 minutes was 0.69% of the dose given, whereas the bile recovery observed was 69.6% of the dose and the assumed liver content was therefore 29.71%. No bromsulphthalein could be detected in the plasma at 210 minutes and the percentage of dye in the blood has been taken to be zero. The amount of dye recovered in the bile observed was 75.23% of the dose given and therefore the assumed liver content is 24.77%.

Summary

The effect of Sodium Taurocholate on the bromsulphthalein distribution curve was only tested in one

animal (dog 438) who was given 11.55 mg./Kg. of bromsulphthalein instead of 5 mg./Kg. body weight. When Sodium Taurocholate was given 45 minutes after bromsulphthalein, the bile volume was noted to increase markedly and there was an immediate increase in the amount of bromsulphthalein. In other words, it would appear as if the increased bile volume rate was associated with an increased excretion of bromsulphthalein although this may not be strictly true, since it may merely represent dye washed out from the dead space within the polythene cannula and the bile duct system inside and outside the liver. The general distribution pattern, however, of the disappearance of bromsulphthalein would appear to agree with that expected from the mathematical model.

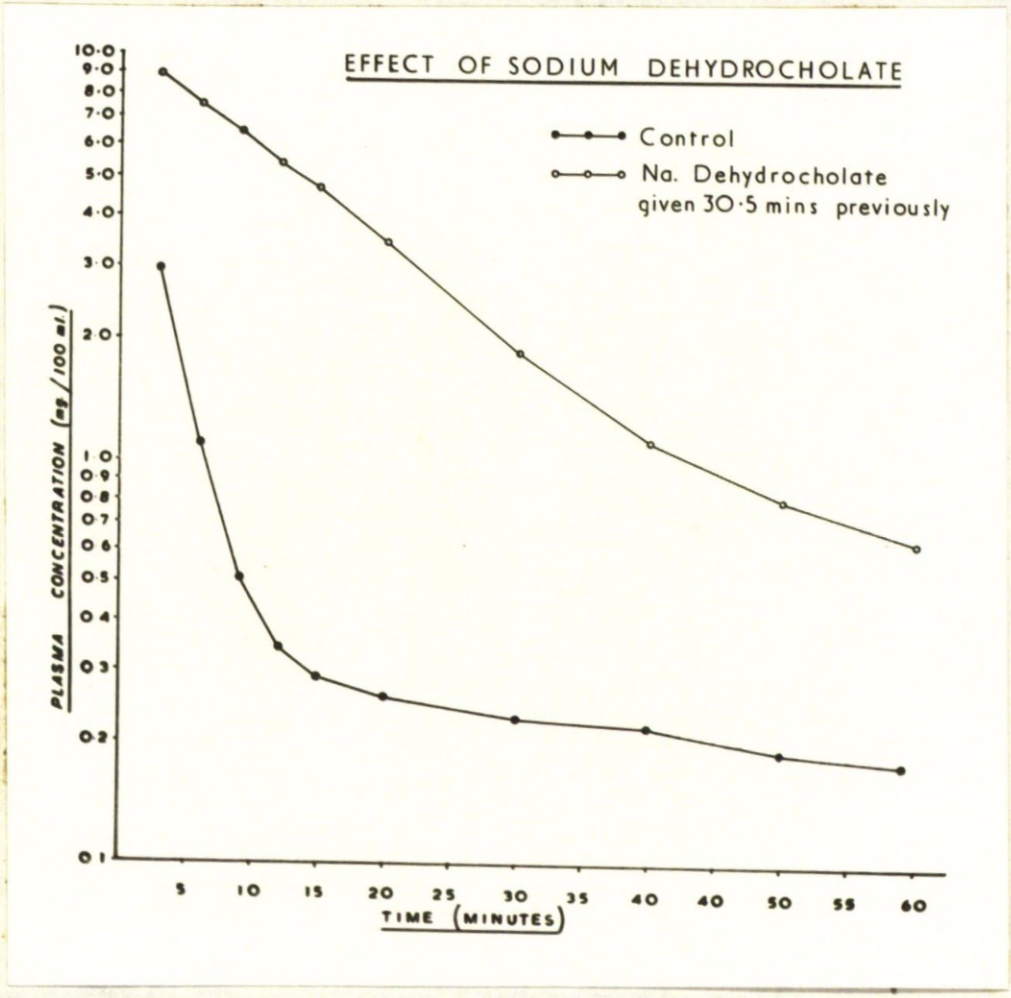
The Effect of "Dehydrocholin" (Sodium Dehydrocholate.).

This substance, which is a well-known choleretic agent has been used to determine its effect on the bromsulphthalein distribution curve. Figure 80 shows the effects of sodium dehydrocholate when given 30.5 minutes prior to a standard dose of bromsulphthalein, and the normal disappearance curve in the same animal taken a few weeks previously.

Figure 80

The effect of Sodium Dehydrocholate (Dehydrocholin").

The effect of Sodium Dehydrocholate given 30.5 minutes prior to the injection of a standard dose of bromsulphthalein, (5 mg./Kg. body weight) is shown. It is compared with the normal disappearance curve of this animal which had been taken some time previously. The typical, double exponential curve of the control specimen appears to have only a single exponential phase when sodium dehydrocholate has been given prior to bromsulphthalein. It may be that the initial rapid phase is unduly prolonged here and towards the end of the period of observation the "bend" portion is only just being approcahed or reached.

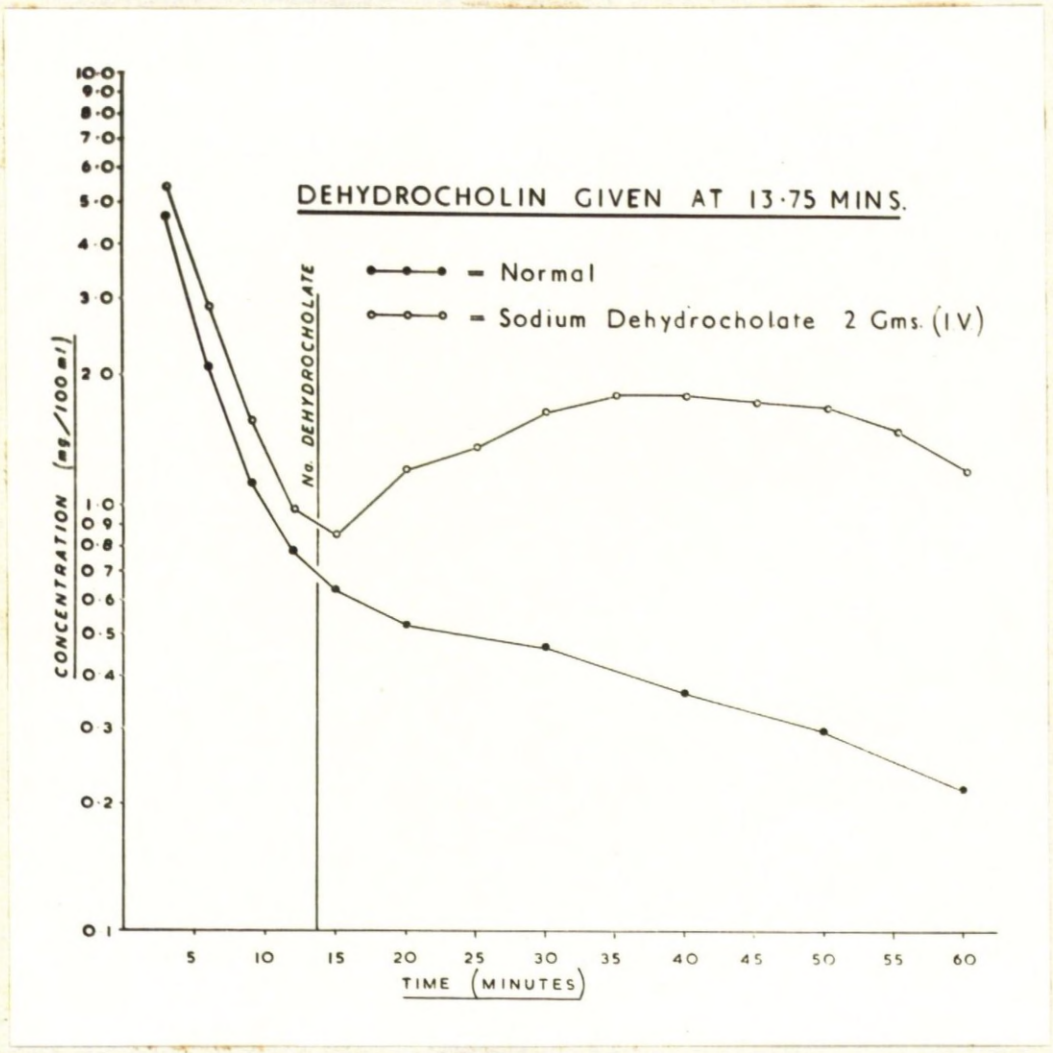

EFFECT OF SODIUM DEHYDROCHOLATE
Control
Na. Dehydrocholate
given 30·5 mins previously
PLASMA CONCENTRATION (mg/100 ml)
10·0
9·0
8·0
7·0
6·0
5·0
4·0
3·0
2·0
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
5
10
15
20
25
30
35
40
40
50
55
60
TIME (MINUTES)

The normal, disappearance of bromsulphthalein follows the typical, double exponential pattern. The graph following sodium dehydrocholate given 30.5 minutes previously appears to be abnormal being represented by a single exponential type of disappearance curve. This may be due to the fact that the initial phase is so prolonged that all the observed concentrations during the 60 minute observation period are virtually on this line. It is unlikely that this can represent the second slower phase, because the initial concentration of bromsulphthalein is so high at three minutes that even allowing time for mixing, there can be virtually no time for dye to be taken up by the liver, prior to the first observed concentration. It is interesting to note that here we have what appears to be a single, exponential curve when the logarithm plasma concentrations are plotted, compared with the normal, double exponential curve obtained in the same animal.

Figure 81 shows the effect of "Dehydrocholin" given 13.75 minutes after an injection of bromsulphthalein. The normal disappearance of bromsulphthalein is also plotted and the observed concentrations prior to the injection of "Dehydrocholin", 2 mg. intra-

Figure 81

The effects of "Dehydrocholin"(Sodium Dehydrocholate) 2 gr. given intravenously, 13.75 minutes after an injection of bromsulphthalein (5 mg./Kg.).
This is compared with the normal disappearance curve which had been noted some time previously. After "Dehydrocholin" (Sodium Dehydrocholate) there is an elevated plasma concentration of bromsulphthalein, possibly due to the re-entry of dye into the circulation from the liver.


DEHYDROCHOLIN GIVEN AT 13·75 MINS.
= Normal
= Sodium Dehydrocholate 2 Gms. (IV)
No. DEHYDROCHOLATE
CONCENTRATION (mg/100 ml)
10·0
9·0
8·0
7·0
6·0
5·0
4·0
3·0
2·0
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
5
10
15
20
25
30
35
40
45
50
55
60
TIME (MINUTES)

venously at 13.75 minutes shows a slight difference between the two readings, but the slopes of these portions of the respective graphs appear to correspond. After "Dehydrocholin" at 13.75 minutes, the plasma concentrations are elevated after a short time interval.

In neither of the animals depicted in Figures 80 and 81, was an acute biliary fistula performed so that no indication can be given of the biliary excretion of bromsulphthalein under the conditions of these two experiments. The two following experiments give some indication of what happens since acute biliary fistulae were performed, "Dehydrocholin" being given simultaneously with and subsequent to bromsulphthalein.

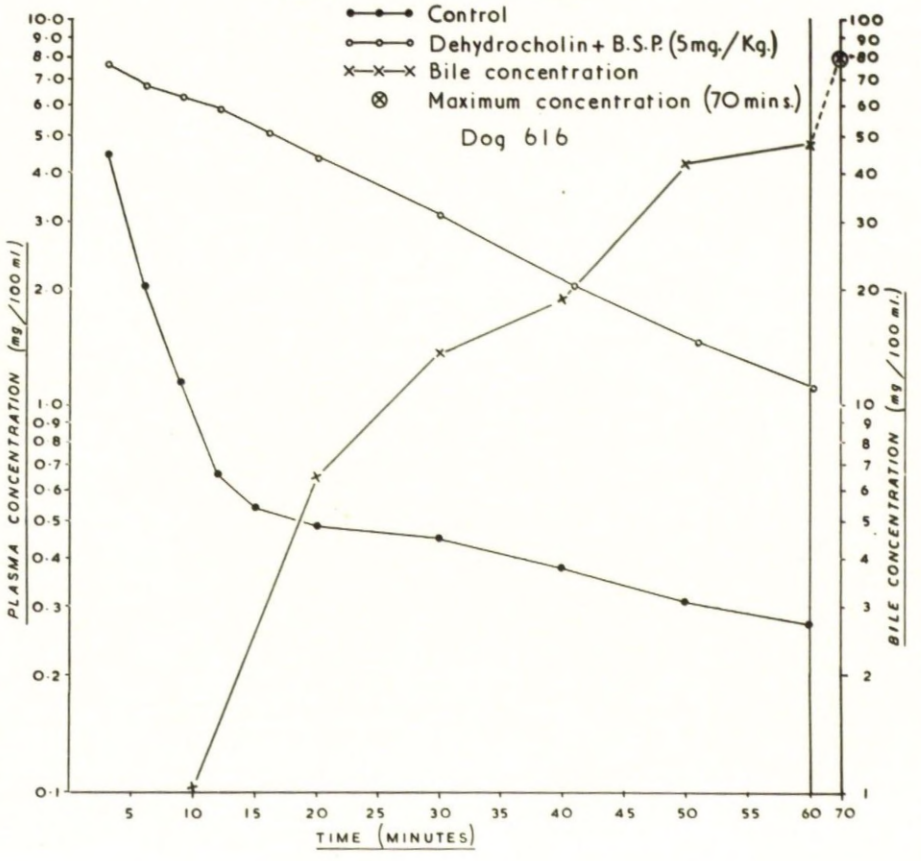
Figure 82 gives some details of an experiment on dog 616, in which sodium dehydrocholate was given simultaneously with bromsulphthalein compared with the control plasma disappearance curve. The pattern of distribution is similar to that noted previously in Figure 80. Once again, the plasma concentration appears to fall on a single straight line after sodium dehydrocholate is given simultaneously with bromsulphthalein. The logarithm of the bromsulphthalein bile

Figure 82

The normal logarithm plasma concentration disappearance curve is compared with that when "Dehydrocholin" (Sodium Dehydrocholate) and bromsulphthalein have been given simultaneously. The graph is similar to those noted when "Dehydrocholin" (Sodium Dehydrocholate) was given half-an-hour prior to bromsulphthalein, as shown in Figure 80.

Also shown in this Figure is the bile concentration of bromsulphthalein. During the 60 minute observation period, the maximum concentration reached was 47.5 mg./100 mls. and the maximum concentration in this experiment was reached at 70 minutes, when the concentration was 80 mg./100 mls. This should be compared with the concentration noted in the normal acute biliary fistula animal and also in the case of Figure 84, where the bile concentration of bromsulphthalein reached just over 550 mg./100 mls. (before Sodium Dehydrocholate was given)

EFFECT OF DEHYDROCHOLIN WHEN GIVEN SIMULTANEOUSLY WITH BROMSULPHTHALEIN



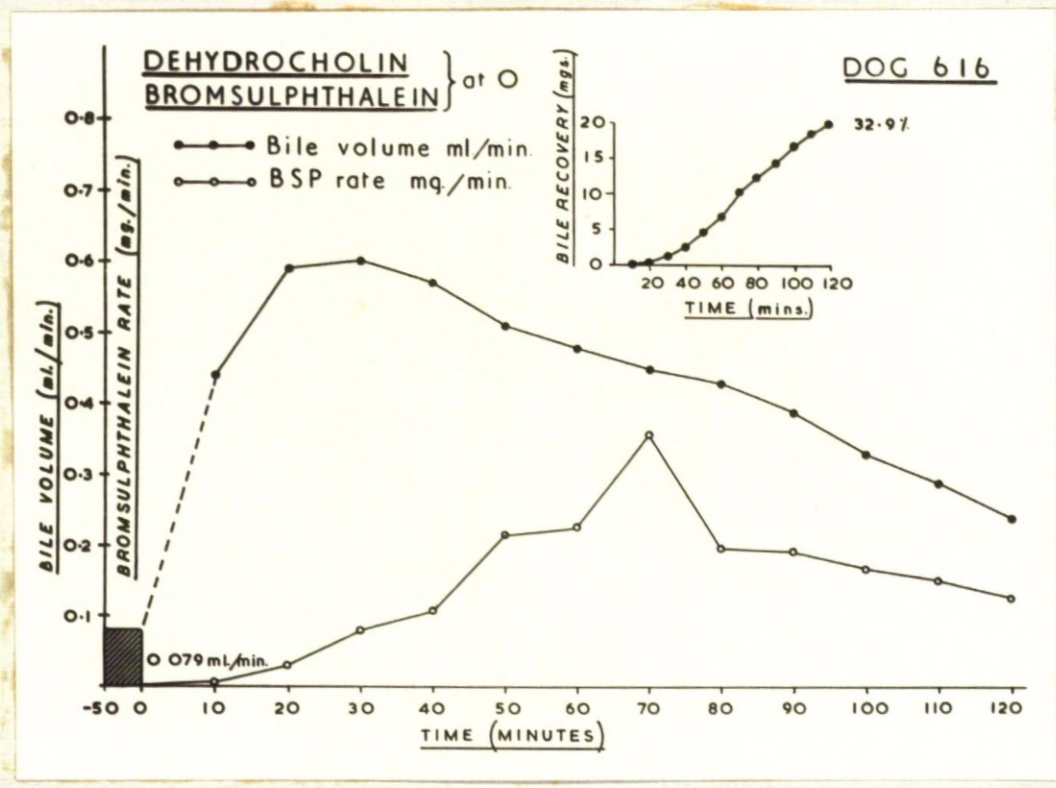
concentration (mg. per 100 mls.) is given in this Figure. The bile concentration gradually increases until it reaches 47.4 mg./100 mls. at 60 minutes. The maximum concentration was reached at 70 minutes, when it was 80 mg./100 mls. If one compares this with the result shown in Figure 84, it can be seen that the bromsulphthalein bile concentration is much lower because in the normal acute biliary fistula animal the maximal bromsulphthalein bile concentration is of the order of several hundred mg./100 mls. The amount of bromsulphthalein recovered in bile would be expected to be reduced, unless the compensatory increase in bile volume is sufficient to yield an equal quantity of bromsulphthalein for any given time interval. The actual amount of dye recovered, is the sum of the bile concentration and the actual volume of bile in any given time period.

Other results obtained in dog 616 are shown in the next Figure (83). The control bile volume prior to the injection of bromsulphthalein and "Dehydrocholin" at zero time for a 50 minute period was 0.079 mls. per minute. The bile volume rate (mls. per minute) increased up to a maximum of just over 0.6 mls. per minute, an increase in bile volume

Figure 83

The effect of sodium dehydrocholate ("Dehydrocholin") on the bile volume and bile recovery of bromsulphthalein is shown in this Figure. The bile volume rate (mls./min.) is given and the control rate of bile volume was 0.079 mls./min., whereas following "Dehydrocholin" the bile rate rose to 0.6 mls. per minute. Some of this effect will be due to bromsulphthalein but the main effect will be due to the action of "Dehydrocholin".

The bromsulphthalein recovery rate (in mg. per min.) is given and it can be seen that the recovery of bromsulphthalein is delayed. The actual amount of dye recovered also is reduced, as shown in the top right-hand section of the graph. The percentage recovery of dye is approximately 7.5% at 60 minutes compared with a normal acute biliary fistula animal, where the recovery is usually 50% or over. Indeed, even after two hours after the injection of bromsulphthalein and sodium dehydrocholate together the total recovery of dye was less than a third of the dose given.

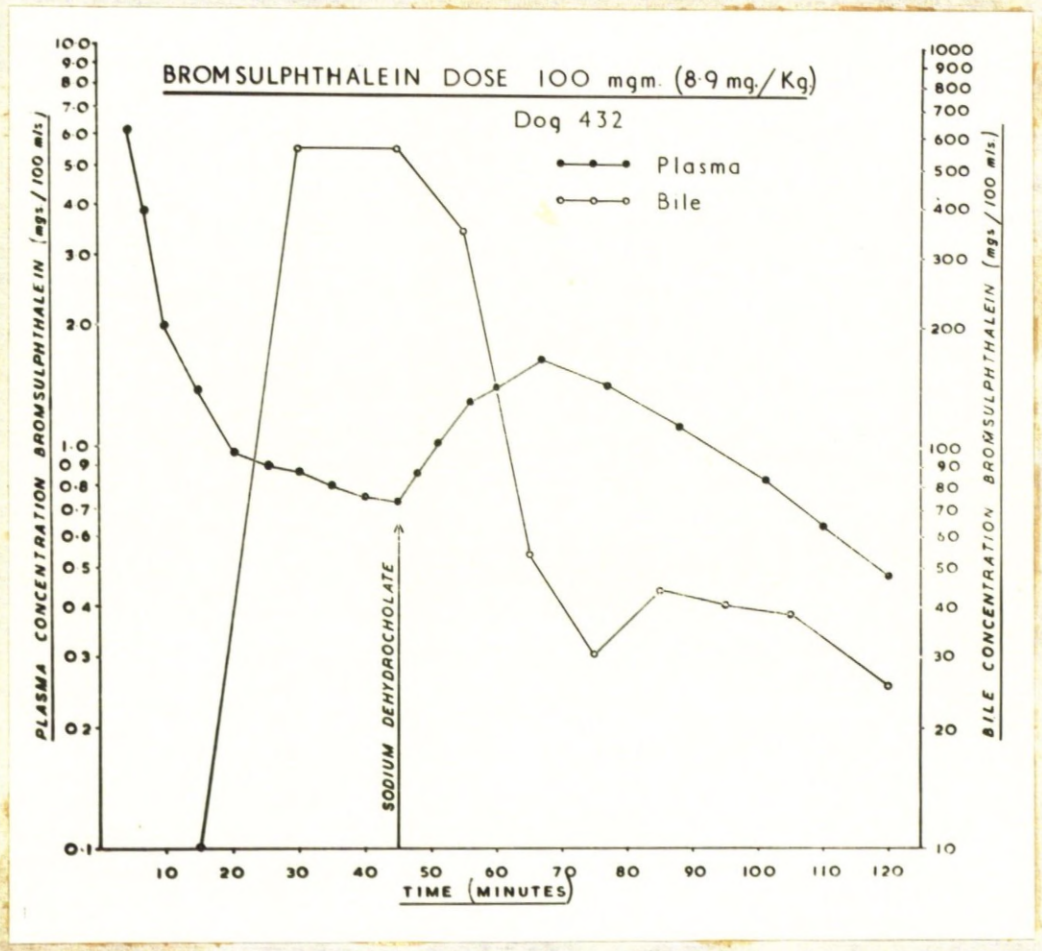

DEHYDROCHOLIN
BROMSULPHTHALEIN
at O
DOG 616
Bile volume ml/min.
BSP rate mg./min.
BILE RECOVERY (mgs.)
32·9%
TIME (mins.)
BILE VOLUME (ml./min.)
BROMSULPHTHALEIN RATE (mg./min.)
0·079 ml./min.
TIME (MINUTES)

rate of approximately eight times the control value. Part of the increase is due to bromsulphthalein but the predominant effect must be due to sodium dehydrocholate. Also shown in the graph is the bromsulphthalein rate in mg. per minute which gradually increases to a maximum, somewhere between 60 and 80 minutes. But the maximal recovery rate of 0.36 mg. per minute which is much lower than has been noted at the maximum in a normal acute biliary fistula dog. In a small insert is shown the percentage of bromsulphthalein recovered. After 60 minutes there is, in fact, less than 7.5% of the dose recovered and even at 120 minutes the amount of dye recovered in the bile is less than a third of the quantity given, which is much lower than the normal biliary fistula animals and even lower than occurred in the abnormal dog (10), which had been subjected to surgical shock and trauma.

An experiment in which sodium dehydrocholate was given 45 minutes after bromsulphthalein given at zero time, is depicted in Figure 84. Bromsulphthalein 100 mg. (8.9 mg./Kg.) was given at zero time and the plasma concentrations observed for a period of 120 minutes. The logarithm plasma concentration shows the typical, double exponential curve until

Figure 84

This shows the effect of sodium dehydrocholate given 45 minutes after an animal had been given a larger (8.9 mg./Kg.) than normal dose, that is 100 mg. of bromsulphthalein.
After the injection of sodium dehydrocholate, the plasma concentration of dye is increased. It is also shown in the graph that the bile concentration of bromsulphthalein after the intravenous injection of sodium dehydrocholate falls markedly.

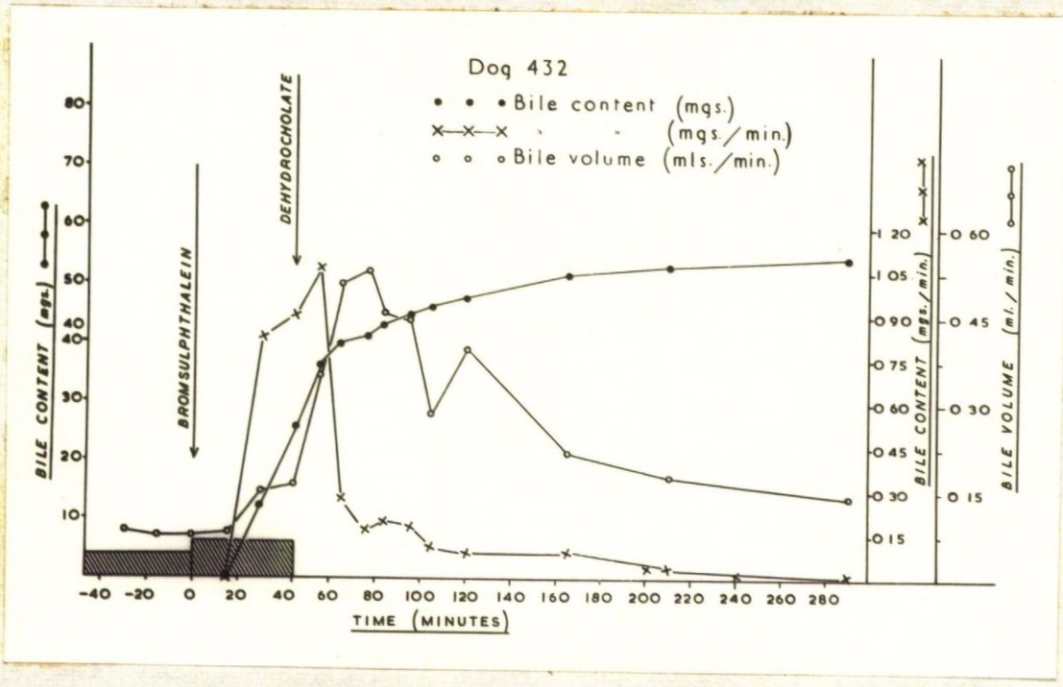

BROMSULPHTHALEIN DOSE 100 mgm. (8·9 mg./Kg.)
Dog 432
Plasma
Bile
PLASMA CONCENTRATION BROMSULPHTHALEIN (mgs./100 mls.)
BILE CONCENTRATION BROMSULPHTHALEIN (mgs./100 mls.)
SODIUM DEHYDROCHOLATE
TIME (MINUTES)
10 20 30 40 50 60 70 80 90 100 110 120

45 minutes, after which sodium dehydrocholate was given. The plasma concentration of the dye then increased to reach a maximum, 20 - 25 minutes after sodium dehydrocholate and then the plasma concentrations gradually fell, but even at 120 minutes the observed plasma concentration was higher than would have been anticipated, if sodium dehydrocholate had not been given. The logarithm bromsulphthalein bile concentration (mg./100 mls.) is of the order of several hundred mg. per 100 mls., which is considerably higher than that depicted in Figure 82, where the maximum was 80 mg./100 mls. Following the injection of sodium dehydrocholate the concentration of bromsulphthalein in the bile fell rapidly and the main effect appeared to be exerted for 30 minutes.

Figure 85 depicts the state of affairs of recovery of dye in a different manner. The average bile volume rate (ml./min.) for the 45 minute control period was 0.08 ml./min. and the choleretic effect of bromsulphthalein itself is seen during the next 45 minutes, when the rate was 0.129 mls. per minute. The bile volume rate following "Dehydrocholin" reached a maximum approximately 30 - 35 minutes later but the bile volume was considerably increased over a 160-

Figure 85

This Figure shows some of the effect on bile content of bromsulphthalein and bile volume following sodium dehydrocholate given at 45 minutes in dog 432, who had been given a 100 mg. dose of bromsulphthalein (8.9 mg./Kg.) at zero time. The average control bile volume rate (mls./min.) is given in the lower left-hand section of the graph. There is a marked choleretic effect following the sodium dehydrocholate injection and following the choleresis the bromsulphthalein content (mg. per min.) is reduced. The actual content of bromsulphthalein was approximately 30% of the dose given by the time sodium dehydrocholate was given and after the initial flushing out of the dead space, 35% of the dose given had been recovered. But for the remainder of the period following sodium dehydrocholate, only 20% of the dose given was recovered in the next 220 minutes.


Dog 432
• • • Bile content (mgs.)
× × × (mgs./min.)
○ ○ ○ Bile volume (mls./min.)
DEHYDROCHOLATE
BROMSULPHTHALEIN
BILE CONTENT (mgs.)
BILE CONTENT (mgs./min.)
BILE VOLUME (ml./min.)
TIME (MINUTES)
-40 -20 0 20 40 60 80 100 120 140 160 180 200 220 240 260 280
10 20 30 40 50 60 70 80
0·15 0·30 0·45 0·60 0·75 0·90 1·05 1·20
0·15 0·30 0·45 0·60

minute observation period, after which it appeared to be returning to the rate reached prior to the injection of sodium dehydrocholate. From the bile content (mg.) also depicted in this graph, it can be seen that at 60 minutes approximately 40% of the dye has been recovered (i.e. fifteen minutes after sodium dehydrocholate had been given) following which there appears to be a decreased rate of excretion of bromsulphthalein, judged by the slow increase during the next 60 minute period, since only 47.5% of the dose was recovered by the end of that time. 280 minutes after the injection of bromsulphthalein, only 55% of the dye given has been recovered. Almost 30% of this dye would have been recovered at 45 minutes if one allows for the immediate "flushing" which would occur following the injection of the choleretic substance (sodium dehydrocholate). Therefore, during a period of approximately 240 minutes, only an extra 25% of bromsulphthalein has been excreted in the bile. The bile content in milligrammes per minute which has been recovered during the 10 or 15 minute periods of observation shows that the bile content rate, prior to the injection of sodium dehydrocholate, had reached over 0.8 mg. per minute. It reached a maximum 10 minutes after the injection of

sodium dehydrocholate when the level was 1.05 mg. per minute. After a ten-minute delay period the choleretic effect of sodium dehydrocholate was marked and it was found that the actual amount of bromsulphthalein recovered fell. Even allowing for the compensatory increase in bile volume it can be seen that the sum of these two yield a smaller recovery of bromsulphthalein in the bile and it is depicted graphically.

Summary

The effect of sodium dehydrocholate ("Dehydrocholin") a well-known choleretic agent appears to have a profound effect on the bromsulphthalein disappearance curve. When given before bromsulphthalein it would appear to be taken up preferentially by the liver cell, since the initial rapid phase of disappearance of bromsulphthalein is unduly prolonged and during the 60-minute observation period, the "bend" has not apparently been reached. When given after the initial rapid phase of bromsulphthalein disappearance curve has taken place, there is an increase in the plasma concentration of bromsulphthalein, presumably due to a return of dye into the blood stream from the liver.

It has also been shown that when sodium dehydrocholate is given simultaneously with bromsulphthalein

the plasma concentration of dye is elevated. In the experiments in which the bile recovery of dye was observed, it has been found that the concentration of dye in the bile is reduced compared with that expected in the normal animal. This effect may be more apparent than real, due to the relatively large dose of sodium dehydrocholate given (2 grammes of the active substance in each dog). It has been shown that the actual amount of dye recovered during the time period of observation is reduced, only 7½% having been recovered in 60 minutes when bromsulphthalein is given simultaneously with sodium dehydrocholate (compared with the normal acute biliary fistula animals where the dye recovery is usually 50% or more). Even two hours after the injection of these two substances together, the total recovery of bromsulphthalein was less than one-third of the dose given.

The effect of Benemid

Probenecid (di-n-propylsulphamyl benzoic acid; benemid; Merck, Sharpe and Dohme) was used by Blondheim (1955) because he hoped it would altogether eliminate the renal loss of bromsulphthalein and so enhance the accuracy of the popular bromsulphthalein retention test. His expectation was reasonable, since it had been shown that benemid inhibited the renal excretion of many substances (Beyer, 1950, 1954). Benemid also inhibits the re-absorption of uric acid by the renal tubules and it has now a wide use in the treatment of gout (Beyer, Russo, Tillison, Miller, Verwey and Gass, 1951; Gutman and Yu, 1955). According to Beyer et al (1951) it is very slowly metabolised in the dog and benemid is still present in the plasma for forty-eight hours following a single dose. As has been mentioned previously, benemid is a definite hydrocholeretic agent and this has been noted in Table 12.

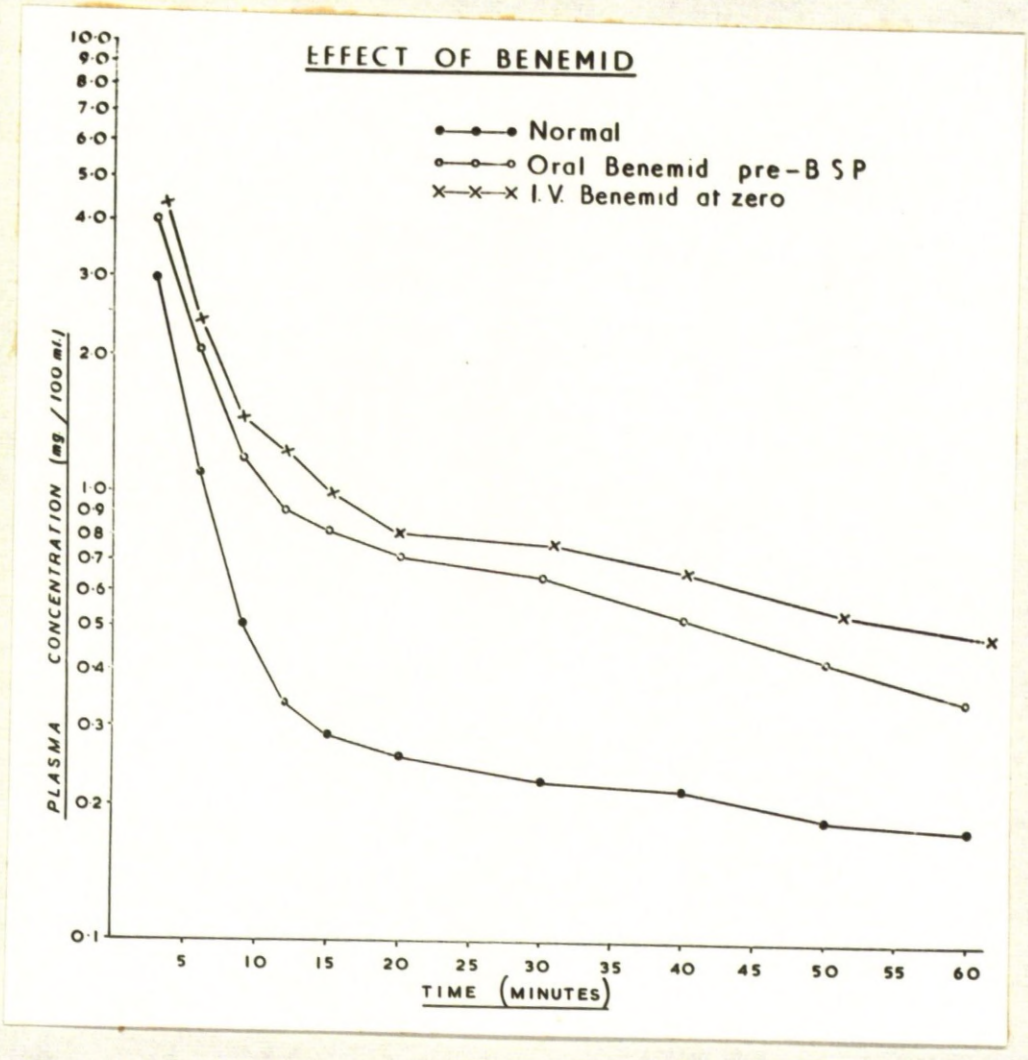
Figure 86 shows the effect of benemid given prior to bromsulphthalein and given intravenously at the same time. The control disappearance curve of bromsulphthalein is given and this was obtained on the 23rd of June, 1958, when the animal weighed 10 Kgs.

Figure 86

The effect of benemid

The normal disappearance graph of bromsulphthalein is given and the effect of oral benemid, 1 gram given daily for three days on the disappearance of bromsulphthalein (5 mg./Kg.) from the plasma on the third day is shown.

Also shown is the effect of intravenous benemid (60 mg.) given at zero time, with bromsulphthalein. Both curves following benemid are elevated, compared with the normal disappearance graph, and the second slower phase appears to be particularly affected.


EFFECT OF BENEMID
Normal
Oral Benemid pre-B S P
I.V. Benemid at zero
PLASMA CONCENTRATION (mg / 100 ml.)
10·0
9·0
8·0
7·0
6·0
5·0
4·0
3·0
2·0
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
5
10
15
20
25
30
35
40
45
50
55
60
TIME (MINUTES)

and received 50 mg. of bromsulphthalein. The second curve shows the effect of oral benemid, 1 gr. being given on the 30th June, 1958, 1 gr. on the 1st July and 1 gram on the 2nd of July. Later on the 2nd of June the bromsulphthalein disappearance curve was taken when the animal weighed 9.85 Kgs. and was given a dose of 50 mg. of bromsulphthalein, which is slightly above 5 mg./Kg., but the raised plasma concentrations are well outside the expected level for such a small increase in the dose of bromsulphthalein. The third disappearance curve depicted, shows the result of an experiment carried out two months later, on the 3rd of September, 1958, when the animal weighed 11.7 Kgs. and was given a 5 mg./Kg. dose of bromsulphthalein. At the same time 60 mg. of benemid was given which is a little over 5.1 mg./Kg. body weight. It can be seen that the observed plasma concentrations at all corresponding times are higher than those of the normal disappearance graph. The disappearance graphs in which benemid has been given, reveal graphs in which the second slower phase is outside the normal accepted limits for an intact animal. The effect of oral benemid appears to be satisfactory, although a large quantity of the substance was given, because at this

time its effects on bromsulphthalein disappearance were uncertain and, indeed, unknown.

Figure 87 shows the effect of benemid given 20 minutes after a standard 5 mg./Kg. dose of bromsulphthalein. The control disappearance graph is shown for this animal and it can be seen that the second disappearance graph prior to the injection of benemid closely corresponds to the observed concentrations of the control. At 20 minutes, benemid, 1.2 grams (100 mg./Kg.) was given intravenously and immediately following this the plasma concentration was found to be elevated. After the initial raised concentration, the subsequent observed concentrations appear to fall on a line which closely corresponded to that of the second slower phase of the control curve.

Figure 88 shows the results of consecutive injections of bromsulphthalein. The dog, weighing 10 Kgs. was given 5 mg./Kg. of bromsulphthalein at zero time and the plasma concentrations were observed for 55 minutes and a further 5 mg./Kg. dose of bromsulphthalein was given intravenously at 60 minutes and the plasma concentrations observed up to 115 minutes. The graphical values of the constants,

Figure 87

This shows the effect of benemid given intravenously on the plasma concentrations after the standard dose of bromsulphthalein. The second injection of bromsulphthalein was given 120 minutes after the first. The rise in plasma concentration following benemid is shown. The rate at which the plasma concentration falls, after benemid, appears to be at almost the same rate on the graph. Benemid, 1.2 grams (100 mg./Kg.) was given twenty minutes after the second injection of bromsulphthalein.

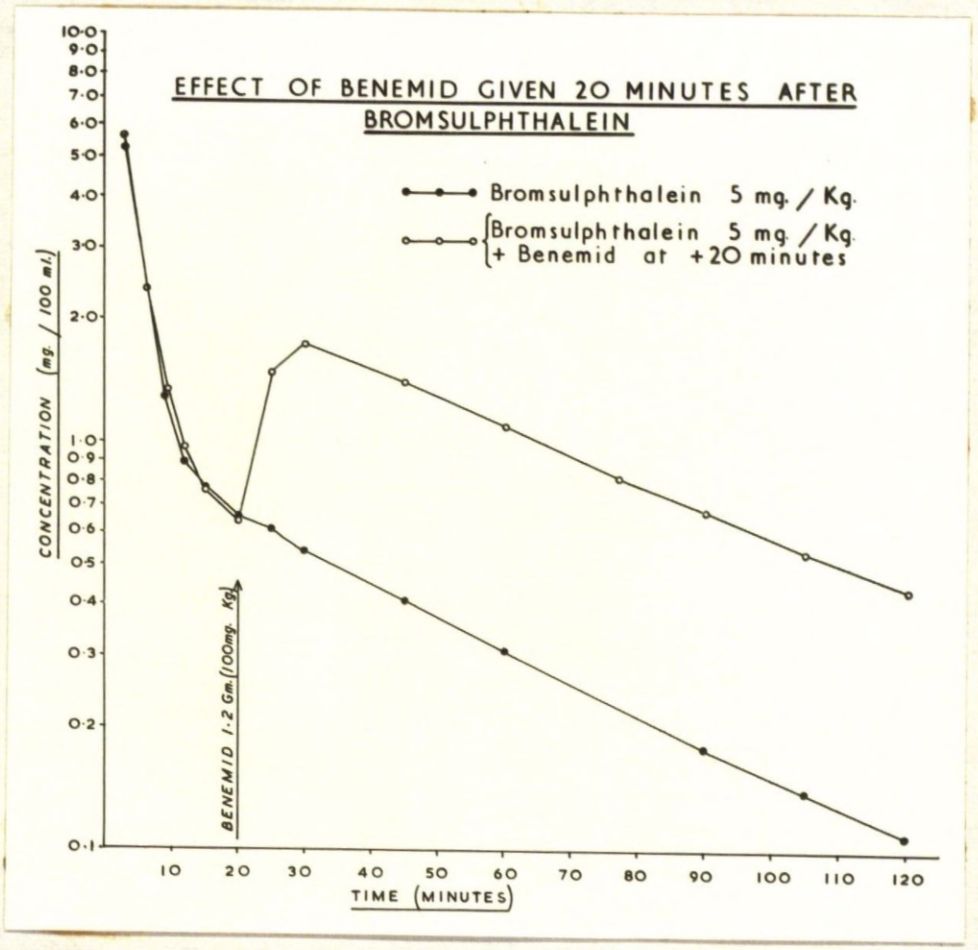
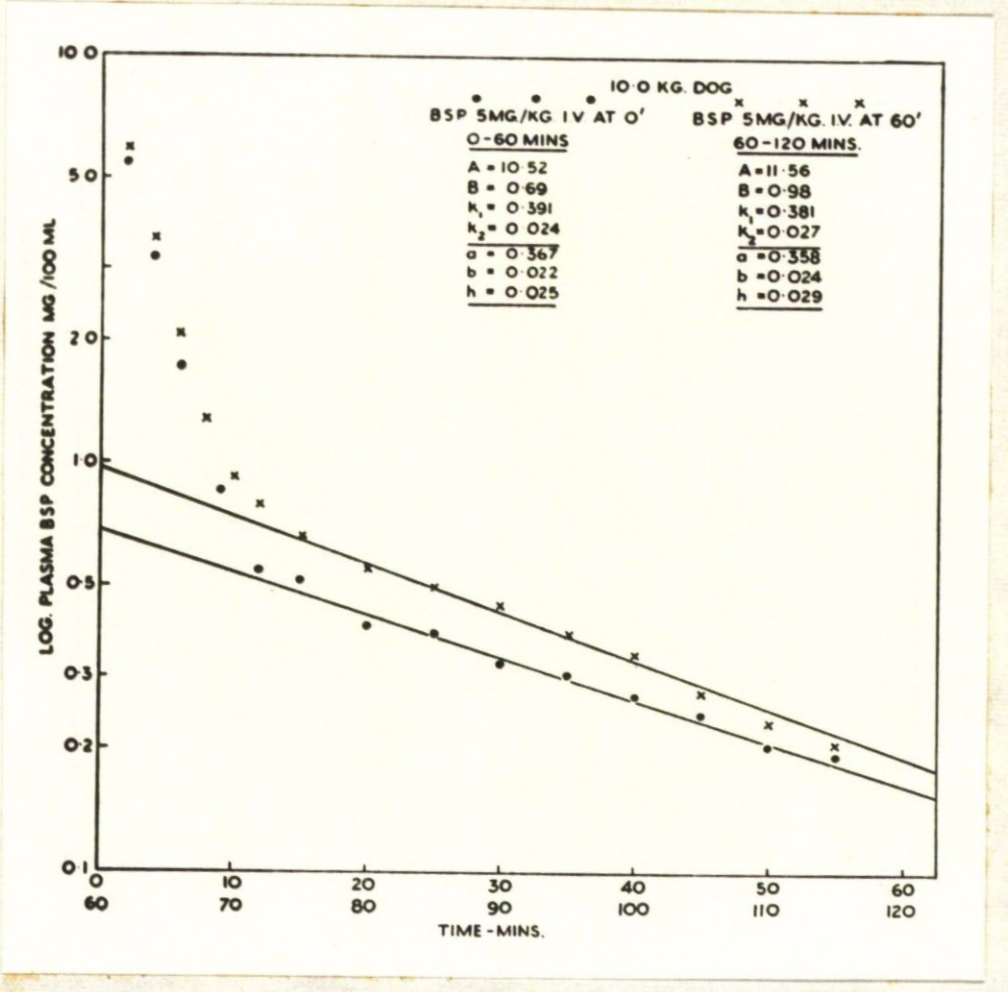

EFFECT OF BENEMID GIVEN 20 MINUTES AFTER BROMSULPHTHALEIN
Bromsulphthalein 5 mg. / Kg.
Bromsulphthalein 5 mg. / Kg. + Benemid at +20 minutes
CONCENTRATION (mg. / 100 ml.)
BENEMID 1·2 Gm. (100mg. Kg.)
TIME (MINUTES)
10·0
9·0
8·0
7·0
6·0
5·0
4·0
3·0
2·0
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
10
20
30
40
50
60
70
80
90
100
110
120

Figure 88

This shows consecutive injections of bromsulphthalein. Two standard doses, (5 mg./Kg.) were given, the second sixty minutes after the first. The values of the constants, k_1 and k_2 are shown for each of the graphs and the constants obtained using the simplified version of the mathematical model have been given at the top of the Figure.


10·0 KG. DOG
BSP 5MG/KG. I.V. AT 0'
0-60 MINS
A = 10·52
B = 0·69
k1 = 0·391
k2 = 0·024
a = 0·367
b = 0·022
h = 0·025
BSP 5MG/KG. I.V. AT 60'
60-120 MINS.
A = 11·56
B = 0·98
k1 = 0·381
k2 = 0·027
a = 0·358
b = 0·024
h = 0·029
LOG. PLASMA BSP CONCENTRATION MG/100 ML
10·0
5·0
2·0
1·0
0·5
0·3
0·2
0·1
0
10
20
30
40
50
60
60
70
80
90
100
110
120
TIME - MINS.

derived as mentioned in the simplified treatment of the mathematical model, are shown in the figure tables. The second curve is raised, since the animal had an initial liver and a plasma load, but it can be seen that the proportionality rates are virtually unaltered. It is therefore assumed that the liver continues to deal with the second dose of dye in the same manner as the first.

In Figure 89, the conditions are the same, but the animal received benemid (100 mg./Kg.) simultaneously with the second dose of dye. Again, the second curve is raised but on inspection the slope of the second part of the curve is now altered and all the proportionality rates are now clearly altered, indicating that the liver did not deal with the second dose of dye as it did with the first.

The visible changes in the plasma concentration graphs, and more particularly the alteration in the proportionality constants, are shown clearly in the next two figures. In Figures 90 and 91 the control disappearance curves were established and ten days later benemid was given intravenously, followed in fifteen minutes by the standard dose of bromsulphthalein. In these experiments, therefore,

Figure 89

This shows consecutive injections of bromsulphthalein. Two standard doses (5 mg./Kg.) were given, the second sixty minutes after the first. Benemid, 150 mg./Kg. accompanied the second dose of bromsulphthalein with a marked effect on both of the constants of the slope k_1 and k_2. Also given are the values of the other constants obtained in a similar manner to those given in Figure 88.

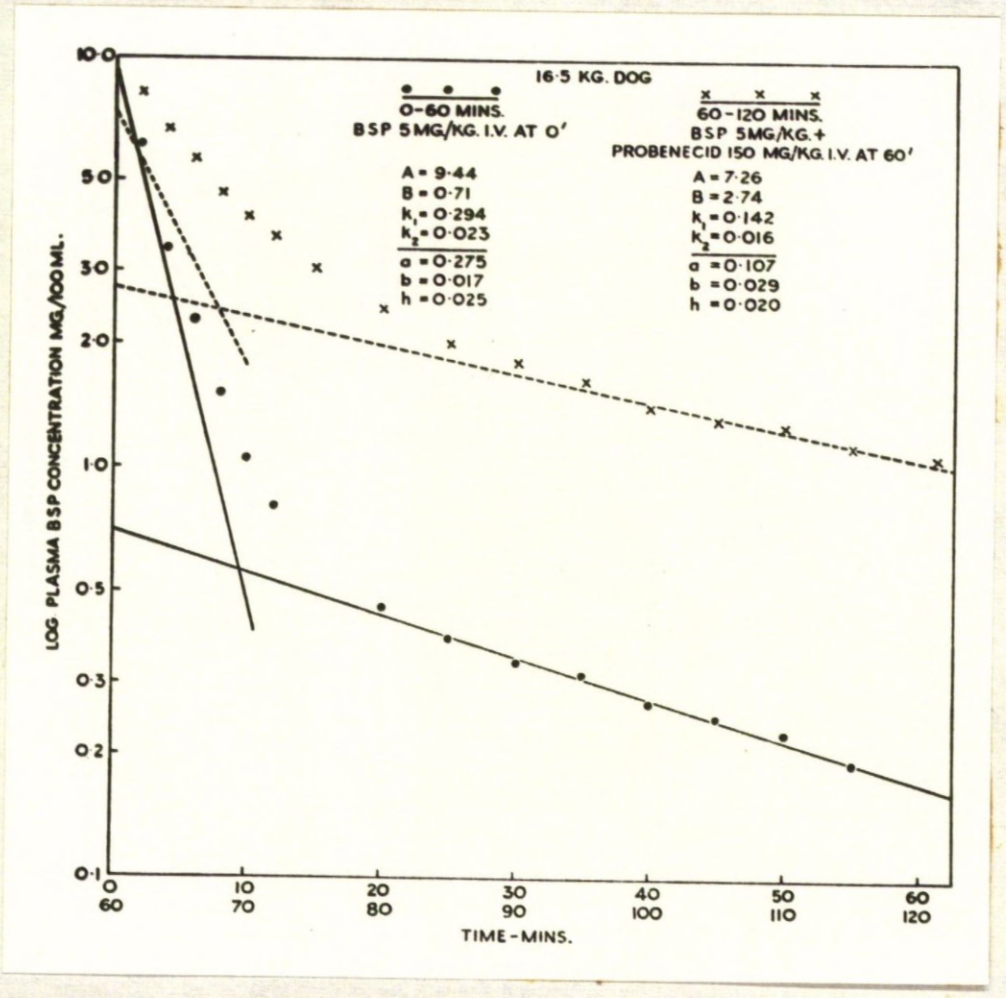

16·5 KG. DOG
0–60 MINS.
BSP 5MG/KG. I.V. AT 0'
A = 9·44
B = 0·71
k_1 = 0·294
k_2 = 0·023
a = 0·275
b = 0·017
h = 0·025
60–120 MINS.
BSP 5MG/KG.+
PROBENECID 150 MG/KG. I.V. AT 60'
A = 7·26
B = 2·74
k_1 = 0·142
k_2 = 0·016
a = 0·107
b = 0·029
h = 0·020
LOG PLASMA BSP CONCENTRATION MG/100 ML.
10·0
5·0
3·0
2·0
1·0
0·5
0·3
0·2
0·1
0 10 20 30 40 50 60
60 70 80 90 100 110 120
TIME–MINS.

Figure 90

This is a graph of the plasma concentration of bromsulphthalein after the injection of a standard dose of dye (5mg./Kg.).
Ten days later, when no initial liver load remained, the animal received benemid (100 mg./Kg.) intravenously, followed in fifteen minutes by bromsulphthalein 5 mg./Kg. Under the condition of this experiment, the reduction in k_2 is seen and a reduction in k_1 found.

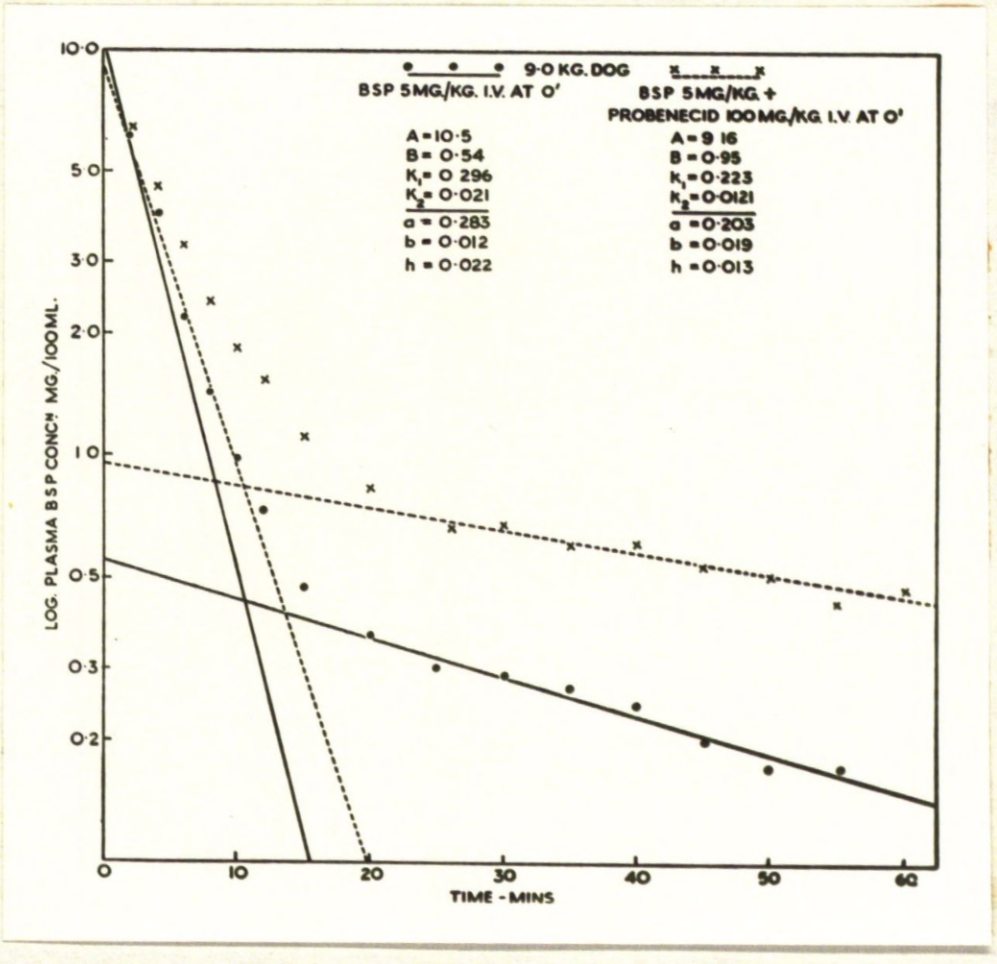
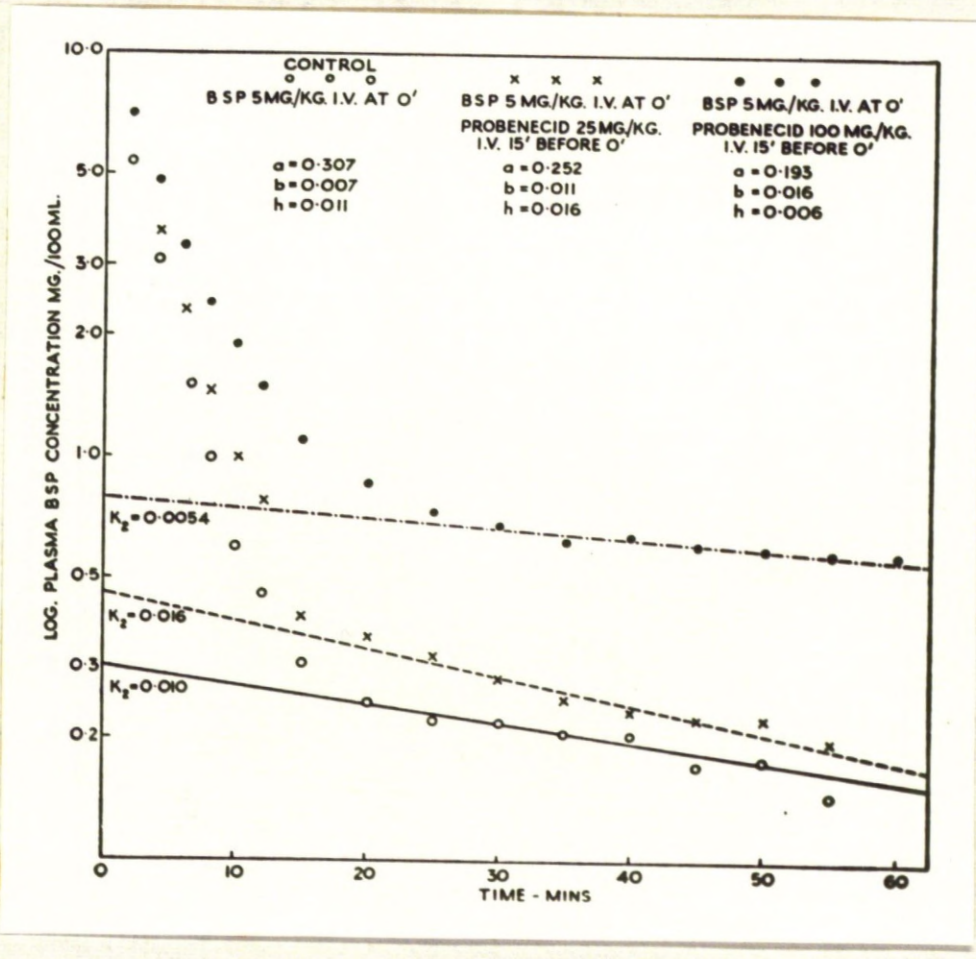

9·0 KG. DOG
BSP 5 MG/KG. I.V. AT 0'
A = 10·5
B = 0·54
K1 = 0 296
K2 = 0·021
α = 0·283
b = 0·012
h = 0·022
BSP 5 MG/KG. +
PROBENECID 100 MG/KG. I.V. AT 0'
A = 9 16
B = 0·95
K1 = 0·223
K2 = 0·0121
α = 0·203
b = 0·019
h = 0·013
LOG. PLASMA BSP CONCN MG./100 ML.
10·0
5·0
3·0
2·0
1·0
0·5
0·3
0·2
0
10
20
30
40
50
60
TIME - MINS

Figure 91

This gives three graphs of bromsulphthalein concentration after the standard dose.
Each experiment was separated by an interval of 14 days. The figure shows the change in k_2 after a dose of 25 mg./Kg. of benemid and 100 mg./Kg. of benemid. The increase of the smaller dose and the decrease after the larger dose are apparent.
The values of the rates or proportionality, "a", "b" and "h" are also shown in the three experiments.


CONTROL
BSP 5MG./KG. I.V. AT O'
a = 0·307
b = 0·007
h = 0·011
BSP 5 MG./KG. I.V. AT O'
PROBENECID 25MG./KG. I.V. 15' BEFORE O'
a = 0·252
b = 0·011
h = 0·016
BSP 5MG./KG. I.V. AT O'
PROBENECID 100 MG./KG. I.V. 15' BEFORE O'
a = 0·193
b = 0·016
h = 0·006
LOG. PLASMA BSP CONCENTRATION MG./100ML.
10·0
5·0
3·0
2·0
1·0
0·5
0·3
0·2
K_2 = 0·0054
K_2 = 0·016
K_2 = 0·010
0
10
20
30
40
50
60
TIME - MINS

there was no existing liver of plasma load during the second determination. In Figure 90, the animal received benemid (100 mg./Kg.), Here, the changes in both the first and second portions of the graph are seen and in this case neither change is due to previous loading of the liver or plasma with dye. The change in the proportionality constants, which have been determined graphically for both curves, are shown.

Figure 91 shows an animal in which control was established and benemid and bromsulphthalein given together at ten-day intervals. The figure shows (i) the initial control curve, (ii) the curve obtained after 25 mg./Kg. of benemid and (iii) the graph after 100 mg./Kg. of benemid. Again the figure shows the changes in the disappearance curves in respect to the control, but it also shows that k_2, the second slope, is not always reduced after benemid and indeed with the smaller dose of 25 mg,/Kg. this slope is increased. The impressions gained from this small series of experiments is that with smaller doses of benemid, there is an increase in the rate of transfer of dye to the bile, per unit load in the liver, that is "h", but it begins to fall when the

dose is increased above this and with a dose of 100 mg./Kg. is below the control. The transfer of dye from the liver to the plasma, that is "b", is immediately affected by benemid. Even with small doses, there is a prompt rise and this continues with increasing dosage but at a slower rate. Transfer of dye per unit plasma load from plasma to the liver, "a", is uniformly decreased as benemid dosage increases. It would appear that benemid, regardless of the dose, decreases the transference of dye from the plasma to the liver and in all doses it increases transfer of dye from the liver to the plasma. In small doses (25 mg./Kg.) benemid increases the transfer rate ("h") from the liver to the bile, but in larger doses, this transfer rate is decreased. Figure 48 which has been inserted mainly to show the effect of benemid on bile flow, also shows the effect of benemid on the excretion of bromsulphthalein in the bile. The output of bromsulphthalein in bile during the 75 minutes which preceded the administration of benemid, varied between 0.525 and 0.413 mg. per minute. After benemid, the rate of excretion of bromsulphthalein in the bile in the first 15 minute sample grows to 0.6 mg. per min., presumably due to the "flushing-out" of the biliary

system of bromsulphthalein, already excreted by the liver cells but not yet removed from the bile canaliculi. For the next 75 minutes, the values range between 0.197 and 0.280 mg. per minute. The main purpose however, of all the experiments in these results, has been the study of a single injection disappearance curve and not continuous infusion experiments.

Summary

The experiments described here show that the process of uptake, storage and excretion of bromsulphthalein is disturbed by the administration of benemid. Benemid appears to affect all phases of dye uptake and removal and since benemid is itself absorbed on protein (Tillson, Schuchardt, Fishman and Beyer, 1954) it may well produce its effect by competitive absorption upon protein in the liver cell, competing with bromsulphthalein, which is absorbed on protein. It has been shown that benemid, given after bromsulphthalein, brings a reflux of dye into the plasma (Figure 87) by displacement of the dye. In these conditions, the proportion of "free" dye in the liver and plasma

would be raised. This in itself does not explain the changes seen in the rates of transfer of dye described above as "a", "b" and "h" and later in the general discussion a hypothesis is put forward which may help to explain the results obtained experimentally. It has been shown that benemid alters the normal mode of dye storage and secretion in a characteristic pattern and the effect of benemid on bromsulphthalein transport was the main purpose of these investigations. By changing one's view point, it may well be that bromsulphthalein alters the manner in which benemid enters and leaves the liver, but this point has not been investigated.

The effect of Biligrafin

Biligrafin (Schering, Berlin), is a substance which is used radiographically for cholecystangiography. It is excreted by the liver so rapidly and in such a concentration that apart from the gall bladder the intra-hepatic and extra-hepatic bile ducts are visualised. As a result of this, routine investigation of the biliary tract, which previously had to be confined to the gall bladder, can now be extended to the bile ducts. In other words, cholecystangiography can be routinely performed, using the radio-opaque dye. Biligrafin causes no local side effects on injection and the general tolerance to biligrafin and Biligrafin Forte is excellent. In cases of liver damage, tolerance to biligrafin is said to be very good. So,far, no absolute contra-indication to the substance has yet been discovered. Similar to bromsulphthalein, special care is necessary with patients suffering from eczema and allergy (Iodine sensitivity, Hay fever, Asthma etc.) and a test for tolerance should be carried out for every case. Biligrafin is made up in two strengths, biligrafin which is a 30% solution of biligrafin and Biligrafin Forte, which is a 50% solution.

In this series of experiments, Biligrafin

Forte has been used and the standard dose given has been 1 c.c./Kg. body weight. Another interesting facet about biligrafin is that the active substance within an oily suspension (Endografin) has been used for hysterosalpingography and it has been noted that sometimes following hysterosalpingography, the bile duct system has also been visualised. Since any extravasation of Endografin reaches the circulation, it must obviously be rapidly taken up by the liver, since it can be noted, during the screening which takes place, during hysterosalpingography, which is completed within 5 - 15 minutes. Since it was apparent that biligrafin must be rapidly excreted by the liver, it was felt worth while carrying out a series of experiments using biligrafin and determining its effect on bromsulphthalein excretion when given at various times before, with or after, bromsulphthalein.

As reviewed in the literature, Sections VII, VIII, IX, there are various normal limits for the standard 5 mg./Kg. dose of bromsulphthalein at various times. It is accepted by all authorities that there is less than 6% of the dose given remaining in the circulation at 45 minutes. At 30 minutes, there

should be less than 10%, at 15 minutes less than 30% and at 5 minutes less than 85%. Judging from the normal scattergram with intact dogs, Figure 39, these respective figures for dogs would be less than 5% at 45 minutes, less than 6% at 30 minutes, less than 10% at 15 minutes and less than 40% at 5 minutes. The series of experiments which have been carried out in the dogs are shown in Table 13. This Table gives the time at which biligrafin has been given, in respect to bromsulphthalein given at zero time. The percentage retention of dye at 5, 15 and 30 minutes is given and the comments as to whether it would be accepted as within the normal human limits, or within the normal range for dogs. As can be seen, the time of giving biligrafin varies in respect of bromsulphthalein from - 240 to + 33 minutes. In each case, the control plasma concentration at the time stated is given. It should be remembered however, and it has already been shown that the individual control graphs may vary considerably within the normal outside limits and an elevated plasma concentration may be observed compared with the control but the result may still be well within the normal limits.

Figure 92 shows the effect of biligrafin given

Table 13

Biligrafin Series

The effect of Biligrafin on the bromsulphthalein disappearance graph. The time of giving Biligrafin in relation to bromsulphthalein given at zero time is stated. A comparison of the percentage retention of dye compared with the control graph at various times is shown, and a comment as to whether or not this result would be considered abnormal in the human or dog.

Accepted normal for human 5 mg/Kg dose :-

5 min.	15 min.	30 min.	45 min.
Less than 85%	Less than 30%	Less than 10%	Less than 6%

For dog these figures would be respectively :-

Less than 40%	Less than 10%	Less than 6%	Less than 5%

The Effect of Biligrafin

Time of giving Biligrafin in respect to Bromsulphthalein at 0 min.	Dog	% retention at 5 min.	% retention at 15 min.	% retention at 30 min.	% retention at 45 min.	Comments
- 240 min.	526	C. 25.5 26.7	3.4 3.2	1.0 1.7	0.8 1.1	Within normal limits (Dog and human)
- 121 min.	516	C. 19.1 32.0	4.6 7.9	3.1 8.1	2.8 4.6	Within normal limits (Dog and human)
- 59 min.	561	C. 22.5 33.5	6.2 9.9	5.4 6.1	3.7 5.3	Within normal limits (Dog and human)

Time of giving Biligrafin in respect to Bromsulphthalein at 0 min.	Dog	% retention at 5 min.	% retention at 15 min.	% retention at 30 min.	% retention at 45 min.	Comments
- 30 min.	561	C. 22.5 48.0	6.2 13.7	5.4 8.9	3.7 8.7	Abnormal throughout in dog. Abnormal 45 min. in human.
- 5 min.	451	C. 16.2 47.0	3.9 18.9	1.9 8.1	1.5 7.6	Abnormal throughout in dog. Abnormal 45 min. in human.
0	516	C. 19.1 62.0	4.6 30.5	3.1 16.0	2.8 14.0	Abnormal throughout in dog. Abnormal 15, 30 and 45 min. in human.
+ $5\frac{1}{2}$ min.	512	C. 24.1 26.1	3.3 15.3	1.6 10.3	1.3 9.6	Abnormal in dog, 15, 30 and 45 min. Abnormal 30 and 45 min. in human.

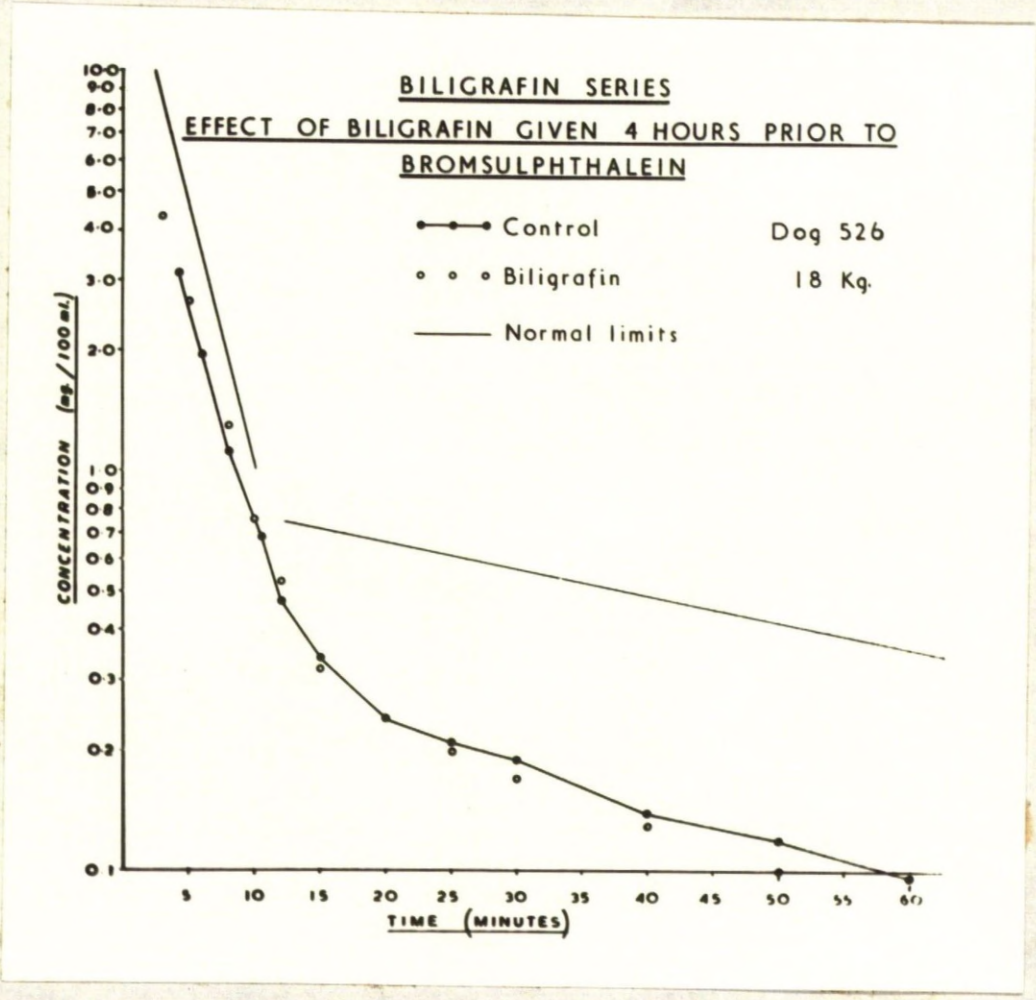
Time of giving Biligrafin in respect to Bromsulphthalein at 0 min.	Dog		% retention at 5 min.	% retention at 15 min.	% retention at 30 min.	% retention at 45 min.	Comments
+ 10 min.	522	C.	38.4	6.8	5.1	4.1	Abnormal in dog, 15, 30 and 45 min.
			37.0	13.4	7.8	8.0	Abnormal 45 min. in human.
+ 10½ min.	347	C.	33.1	5.3	3.1	2.8	Abnormal in dog, 15, 30 and 45 min.
			31.5	14.0	13.8	11.5	Abnormal 30 and 45 min. in human.
+ 20½ min.	537	C.	27.5	7.2	4.1	3.4	Abnormal in dog, 30 and 45 min.
			27.5	5.2	9.3	9.8	Abnormal 45 min. in human.
+ 33 min.	537	C.	27.5	7.2	4.1	3.4	Within normal limits (Dog and human.)
			25.0	3.9	2.2	3.2	

C = Control

Figure 92

Biligrafin Series

Biligrafin was given intravenously four hours prior to bromsulphthalein, and the observed concentrations compared with the logarithm plasma concentration plotted against time. The result in this experiment is compared with the normal control graph.


BILIGRAFIN SERIES
EFFECT OF BILIGRAFIN GIVEN 4 HOURS PRIOR TO BROMSULPHTHALEIN
Control
Biligrafin
Normal limits
Dog 526
18 Kg.
CONCENTRATION (mg/100 ml)
TIME (MINUTES)

4 hours prior to bromsulphthalein. The observed plasma concentrations after biligrafin follow closely those on the control curve. It would therefore appear that biligrafin is excreted within 4 hours, or at least by this time it has no effect on the disappearance of bromsulphthalein from the plasma.

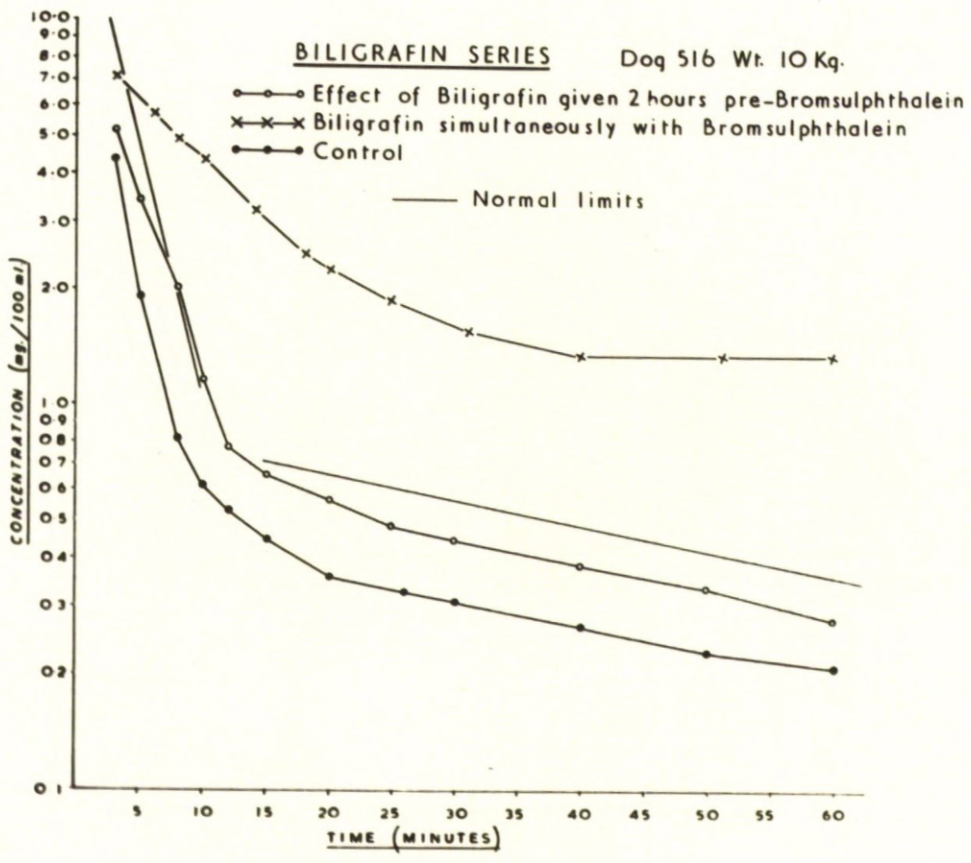
Figure 93 shows the effect of biligrafin in dog 516, in which biligrafin was given 2 hours prior to bromsulphthalein and also shows the results on a separate occasion when biligrafin had been given simultaneously with bromsulphthalein. In both cases, there was no bromsulphthalein in the plasma from previous experiments, since at least a week had elapsed between the experiments. The control curve is given and the lines for the normal limits are also shown. The plasma concentrations observed when biligrafin has been given two hours prior to bromsulphthalein, results in elevated plasma concentration. The overall impression of the results is that the graph falls within normal limits. The effect of bromsulphthalein and biligrafin given together is more marked a double, exponential curve results, but both components are raised compared with the normal and all concentrations are well outside the normal limits.

Figure 93

Biligrafin Series

The effect of biligrafin given two hours prior to bromsulphthalein and also the effect of biligrafin given simultaneously with bromsulphthalein on different occasions compared with the normal control graph for the same dog.

On each occasion, 5 mg./Kg. of bromsulphthalein was given and 1 c.c. of Biligrafin Forte /Kg. body weight.


BILIGRAFIN SERIES
Dog 516 Wt. 10 Kg.
Effect of Biligrafin given 2 hours pre-Bromsulphthalein
Biligrafin simultaneously with Bromsulphthalein
Control
Normal limits
CONCENTRATION (mg/100 ml)
10·0
9·0
8·0
7·0
6·0
5·0
4·0
3·0
2·0
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
5
10
15
20
25
30
35
40
45
50
55
60
TIME (MINUTES)

The results with the dose used in the latter case appeared to be similar to the effect of bromsulphthalein and benemid given together.

Figure 94 gives details of two experiments and the results are compared with the normal bromsulphthalein disappearance curve. Biligrafin has been given respectively at 59 minutes and 30 minutes prior to bromsulphthalein and in this particular animal the effect appeared to be more marked the closer biligrafin was given to the bromsulphthalein injection. Both graphs are outside the normal limits and this occurs in the case of an animal whose own control curve was near the outside limits of normal. The effect, in either case, however, is not as marked as that shown in Figure 93, when biligrafin and bromsulphthalein were given simultaneously.

The next figure in this series (Figure 95), shows the effect of biligrafin given 5 minutes prior to bromsulphthalein, and once again the control disappearance graph and the accepted upper limits of normal are given. The observed bromsulphthalein concentrations on this occasion are a little more variable than has been noted in other instances, but again the graph appeared to consist of exponential

Figure 94

Biligrafin Series

The effect of biligrafin given 59 minutes prior to bromsulphthalein and 30 minutes prior to bromsulphthalein is shown in this graph and compared with the normal control curve

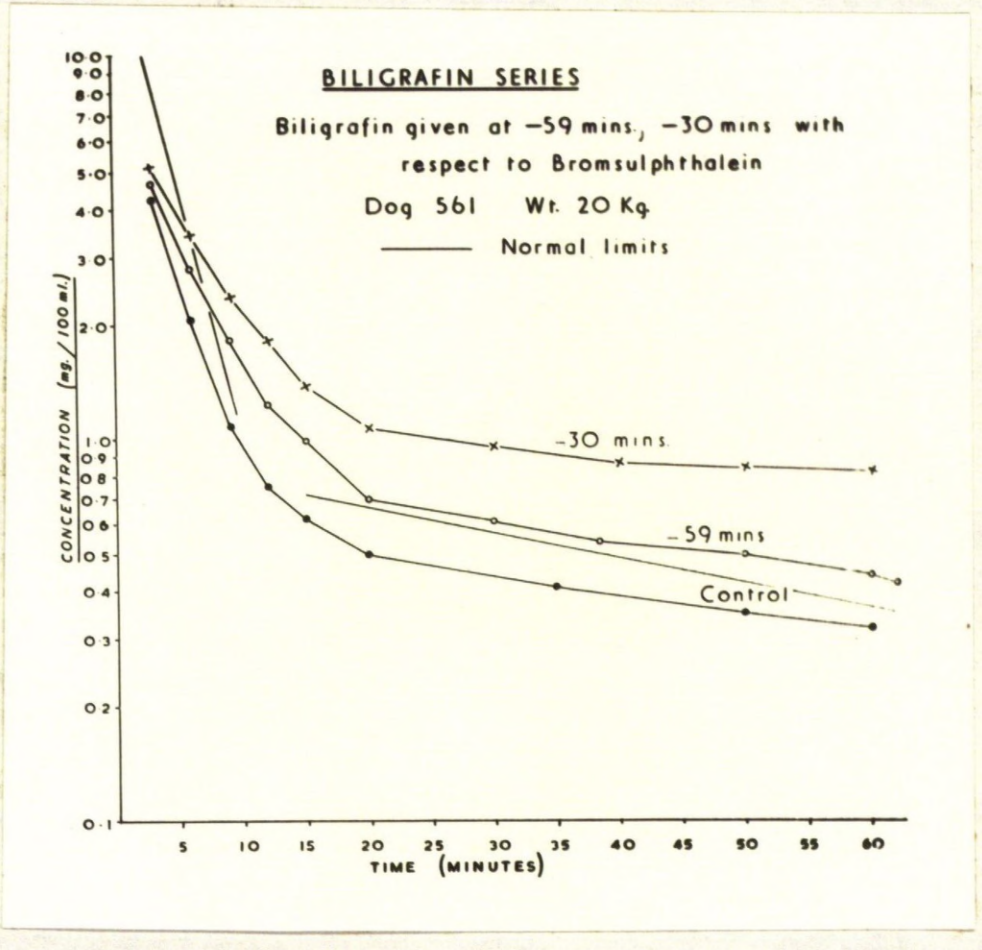
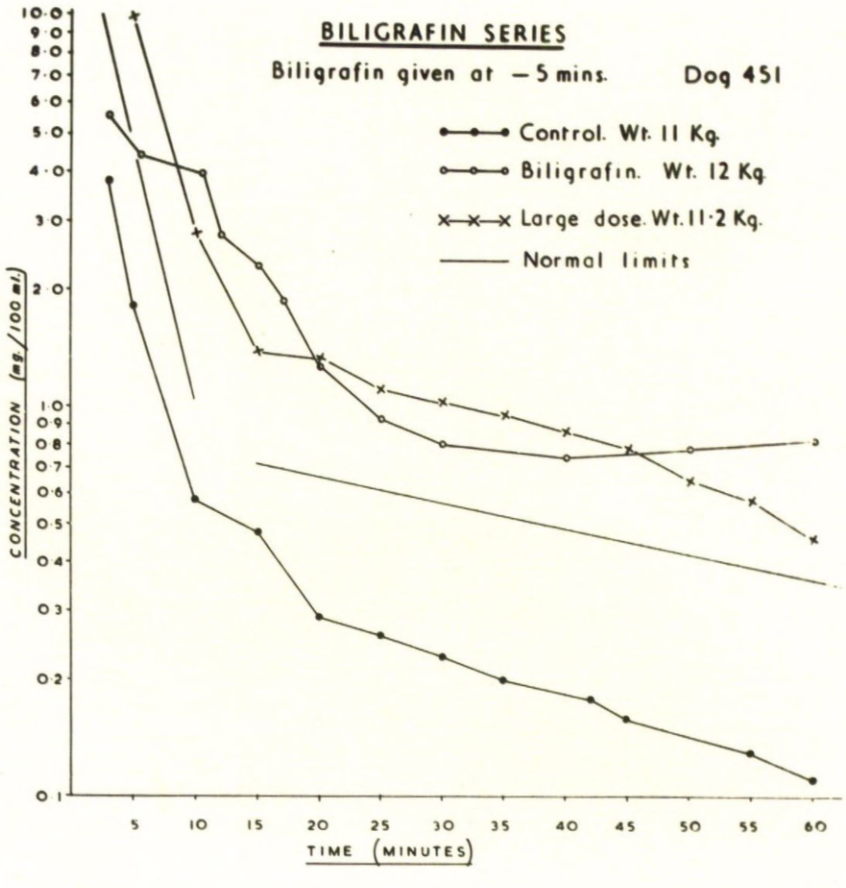

BILIGRAFIN SERIES
Biligrafin given at −59 mins., −30 mins with respect to Bromsulphthalein
Dog 561 Wt. 20 Kg.
Normal limits
−30 mins.
−59 mins
Control
CONCENTRATION (mg./100 ml.)
TIME (MINUTES)

Figure 95

Biligrafin Series

The effect of biligrafin given 5 minutes prior to bromsulphthalein is compared with the normal control curve.
Also shown in this Figure for comparison, is the effect of a large dose of bromsulphthalein (approximately 22 mg./Kg. body weight).


BILIGRAFIN SERIES
Biligrafin given at — 5 mins.
Dog 451
Control. Wt. 11 Kg.
Biligrafin. Wt. 12 Kg.
Large dose. Wt. 11·2 Kg.
Normal limits
CONCENTRATION (mg./100 ml.)
10·0
9·0
8·0
7·0
6·0
5·0
4·0
3·0
2·0
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
5
10
15
20
25
30
35
40
45
50
55
60
TIME (MINUTES)

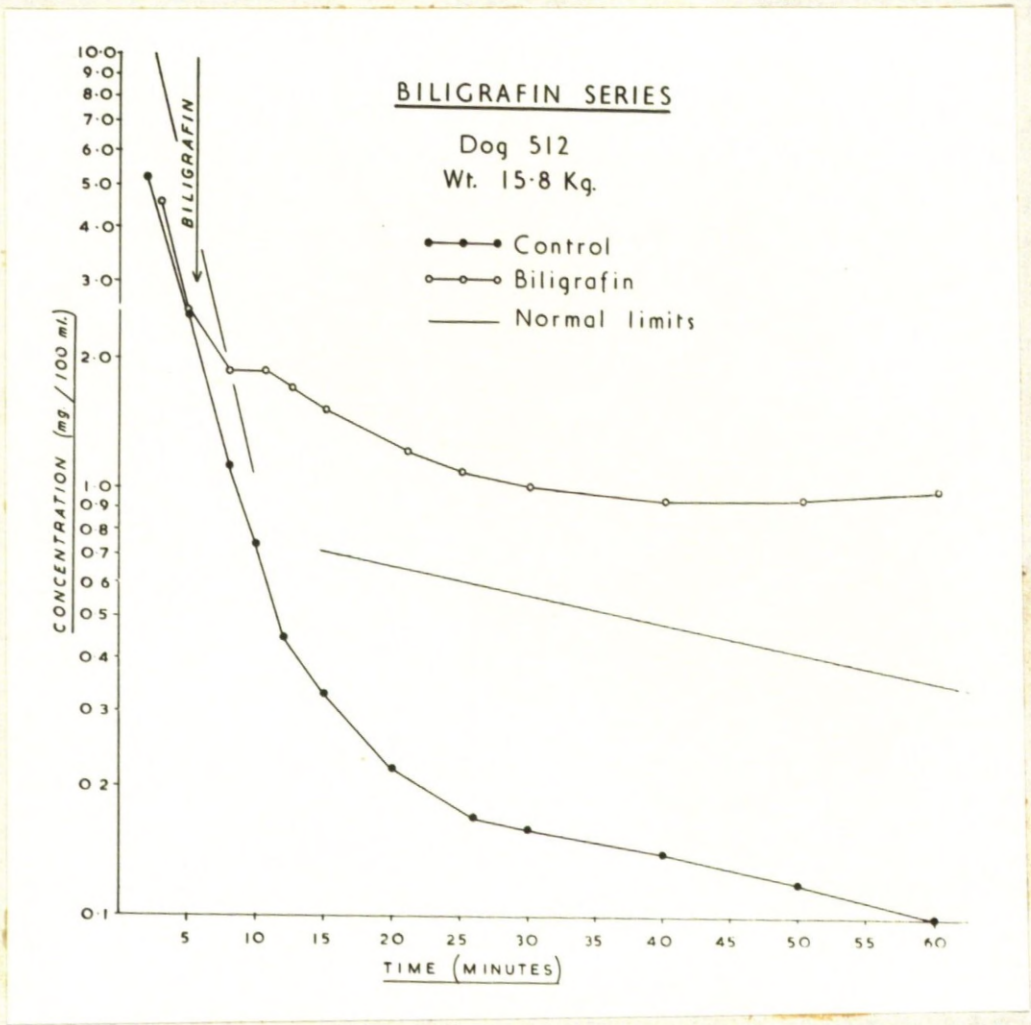
curves. The second phase, however, shows some alteration in the "normal" characteristics in that it tends to be elevated at 50 and 60 minutes as compared with the 30 and 40 minute plasma concentrations. This Figure also shows the effect of a large dose of bromsulphthalein in this animal. The animal weighed 11.2 Kgs. at the time the large dose was given and the animal was given 250 mg. (approximately 22.3 mg./Kg. body weight). There is an initial rapid fall concentration and a second slower phase in which the dye appears to be being excreted quite satisfactorily. There is no evidence of the saturation or straight line phase which was noted in the animal in Figure 51, who was also given a large dose (20 mg./Kg.). It would appear as if biligrafin exerts a competitive effect for some part of the "pathway" used for bromsulphthalein excretion, since the effect of a large dose of bromsulphthalein is in some ways similar to that noted when biligrafin has been given prior to bromsulphthalein.

Figure 96 shows the effect when biligrafin is injected 5½ minutes after a standard dose of bromsulphthalein had been given. It can be seen that on this occasion, the second slower phase appears to have been

Figure 96

Biligrafin Series

This shows the effect of biligrafin given 10 minutes after bromsulphthalein and the result is compared with the normal control disappearance curve. On both occasions, 5 mg./Kg. of bromsulphthalein and 1 c.c. of Biligrafin Forte/Kg. body weight had been given.


BILIGRAFIN SERIES
Dog 512
Wt. 15·8 Kg.
Control
Biligrafin
Normal limits
BILIGRAFIN
CONCENTRATION (mg / 100 ml.)
10·0
9·0
8·0
7·0
6·0
5·0
4·0
3·0
2·0
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
5
10
15
20
25
30
35
40
45
50
55
60
TIME (MINUTES)

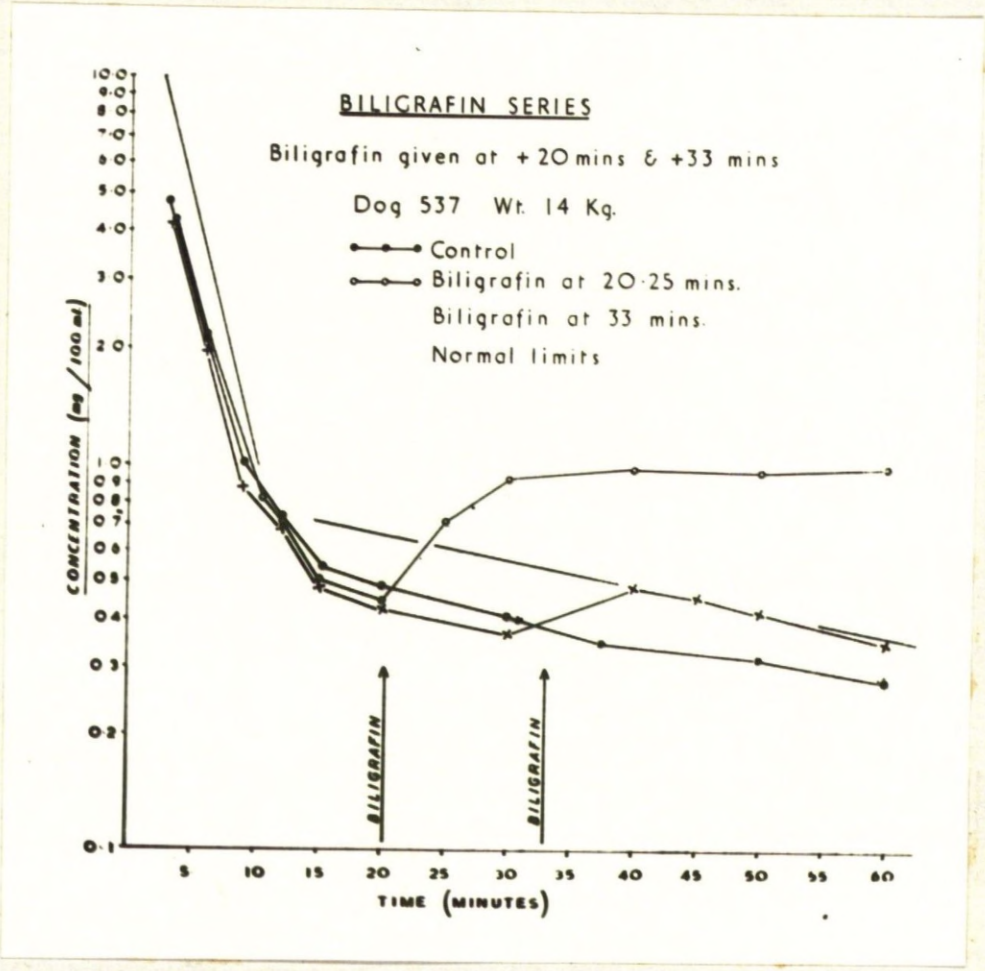
definitely altered and again it is noted that the observed plasma concentrations at 50 and 60 minutes appear to be rising rather than falling, as compared with the 30 and 40 minute observed plasma concentrations. The effect of biligrafin is fairly rapid since the observed plasma concentration 2½ minutes after the biligrafin has been given is considerably raised compared with that which would have been expected in the normal graph.

Figure 97 shows the effect of biligrafin given to a dog at 20.25 minutes and 33 minutes respectively after the bromsulphthalein injection. The normal disappearance curve is given and it should be noted that prior to the injection of biligrafin on each occasion, the observed plasma concentrations correspond very closely to the control value. Following the injection of biligrafin at 20.25 minutes, the plasma concentrations become markedly elevated and this portion of the graph should be compared with Figure 87 which noted the effect of benemid at this time. The effect of biligrafin when given 33 minutes after bromsulphthalein is not so marked, presumably due to the smaller quantity of dye present in the plasma, but despite

Figure 97

Biligrafin Series

This graph shows the effect of biligrafin given 20 minutes and 33 minutes respectively following bromsulphthalein on different occasions and they are compared with the control for this particular animal.
The usual standard doses of bromsulphthalein and biligrafin have been given. The normal limits are also shown.


BILIGRAFIN SERIES
Biligrafin given at +20 mins & +33 mins
Dog 537 Wt. 14 Kg.
Control
Biligrafin at 20-25 mins.
Biligrafin at 33 mins.
Normal limits
CONCENTRATION (mg / 100 ml)
BILIGRAFIN
BILIGRAFIN
TIME (MINUTES)

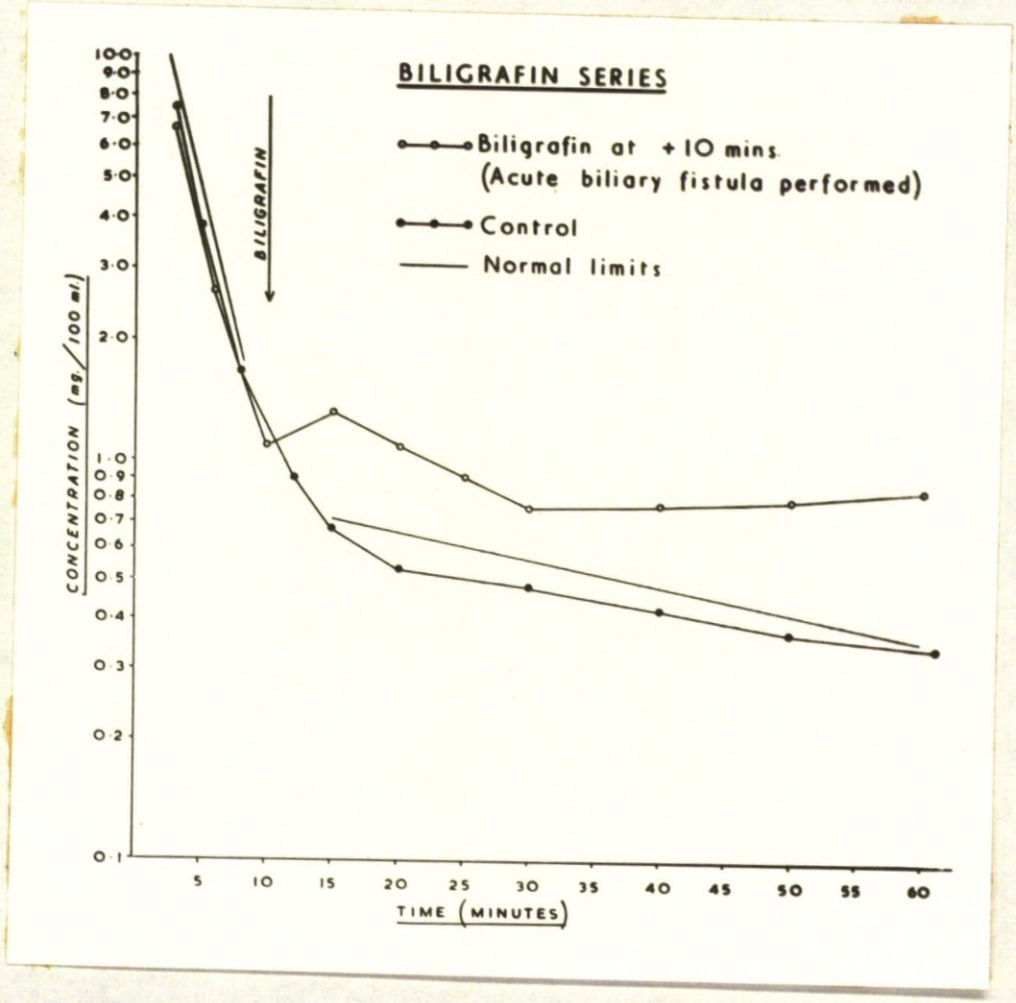
this there is once again a definite elevation compared with the expected slope based on the earlier observed plasma concentrations. In this particular experiment it would appear that following biligrafin and after the peak elevated plasma concentration has been reached, the dye is excreted a little more rapidly than previously (but this is uncertain because there are so few observations at this particular stage of the experiment).

Figure 98 shows the effect of biligrafin in dog 522 who had an acute biliary fistula performed. All the previous experiments had been on the intact dog. The control disappearance curve of bromsulphthalein is given as well as the normal limits. Biligrafin was given 10 minutes after the injection of bromsulphthalein and once again it can be seem that the observed concentrations prior to biligrafin being injected are in good agreement. There is the elevated plasma concentration, followed by a rapid phase of disappearance and this is followed by a slower phase of disappearance, after which there is apparently a further increase in the amount of dye returned to the plasma. This animal was, in fact, observed for a longer period than 60 minutes (the

Figure 98

Biligrafin Series

The effect of biligrafin given 10 minutes after an injection of bromsulphthalein compared with the normal control curve. This animal had an acute biliary fistula performed and therefore the bile recovery of dye has been observed.


BILIGRAFIN SERIES
Biligrafin at +10 mins
(Acute biliary fistula performed)
Control
Normal limits
BILIGRAFIN
CONCENTRATION (mg./100 ml)
100
9·0
8·0
7·0
6·0
5·0
4·0
3·0
2·0
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
5
10
15
20
25
30
35
40
45
50
55
60
TIME (MINUTES)

time period in all other animals in this series) but, for the sake of comparison, the graph has been presented in this form.

Figure 99, however, shows the observed concentrations for a period of 120 minutes plus the final reading obtained at the end of the experiment (177 minutes). The impression that the plasma concentration was being increased due to a return of dye to the plasma is confirmed, since during the 60 - 120 minute phase, the plasma concentration gradually rises from 0.84 to 1.0 and even at 170 minutes the plasma concentration is still 0.98 mg./100 mls.

Figure 100 shows some of the observed effects on the bile in dog 522. The bile concentration in mg./100 mls. is shown and at ten minutes this is of the order of 150 mg./100 mls. but, following biligrafin increases rapidly to 220 mg./100 mls., following which there is a fall to between 80 and 100 mg./100 mls. until about 165 minutes, after which there is a slight increase in the concentration of bromsulphthalein in mg./100 mls. A comparison is made of the percentage recovery of bromsulphthalein in the bile in this animal with one of the normal

Figure 99

Biligrafin Series

The logarithm plasma concentrations obtained in dog 522, with an acute biliary fistula.
Biligrafin was given 10 minutes after bromsulphthalein. The results are shown for the full duration of the experiment, which lasted for 177 minutes.

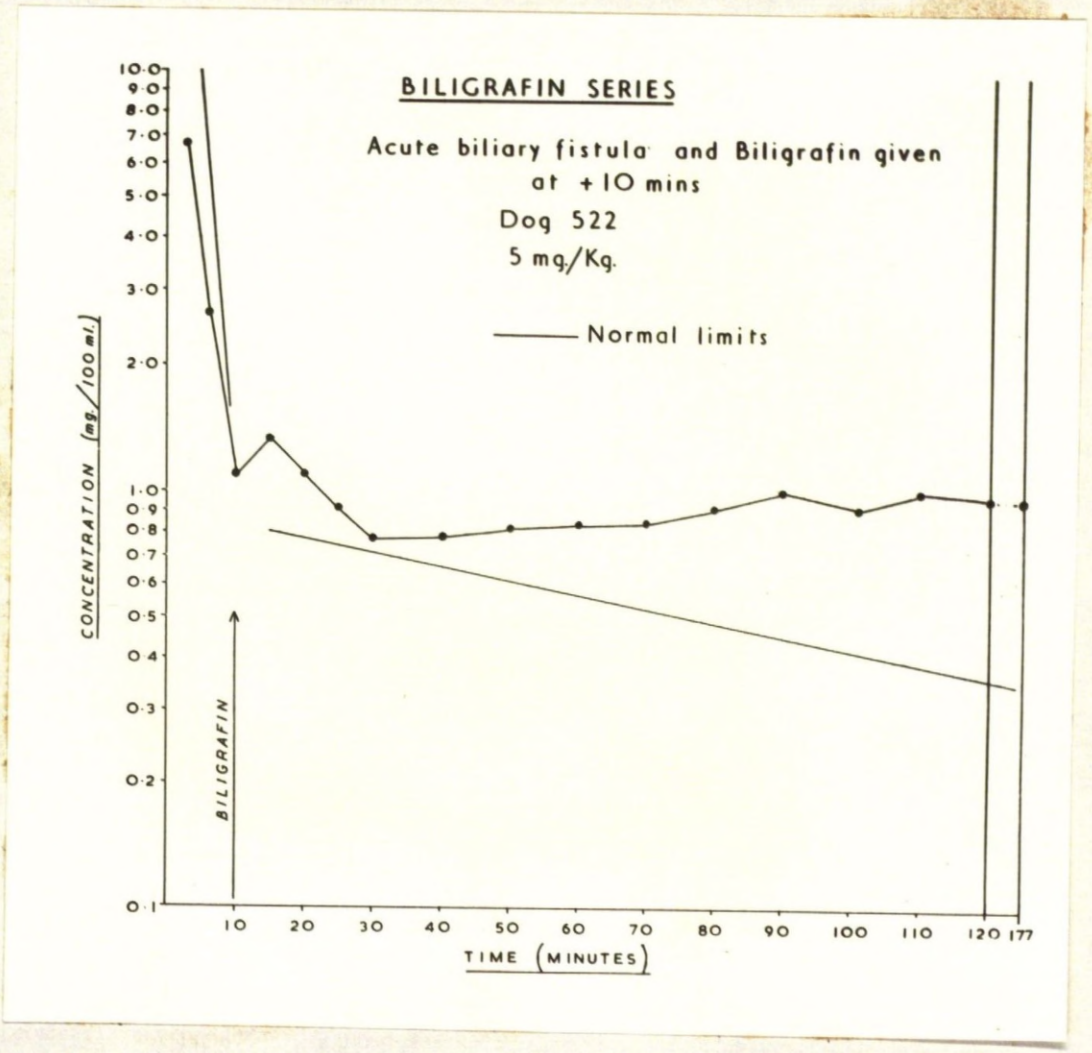

BILIGRAFIN SERIES
Acute biliary fistula and Biligrafin given at +10 mins
Dog 522
5 mg./Kg.
Normal limits
CONCENTRATION (mg./100 ml.)
10·0
9·0
8·0
7·0
6·0
5·0
4·0
3·0
2·0
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
BILIGRAFIN
10
20
30
40
50
60
70
80
90
100
110
120
177
TIME (MINUTES)

Figure 100

Biligrafin Series

The bile concentration of bromsulphthalein and the percentage of dye recovered in the bile of dog 522 after a 5 mg./Kg. dose of bromsulphthalein had been given, is shown. The animal, who had an acute biliary fistula operation performed, was given biligrafin 1 c.c./Kg. body weight, 10 minutes after bromsulphthalein. The percentage of bromsulphthalein in the bile in this animal is compared with the normal recovery in dog 2 (an acute biliary fistula animal used in Section A of the Results).

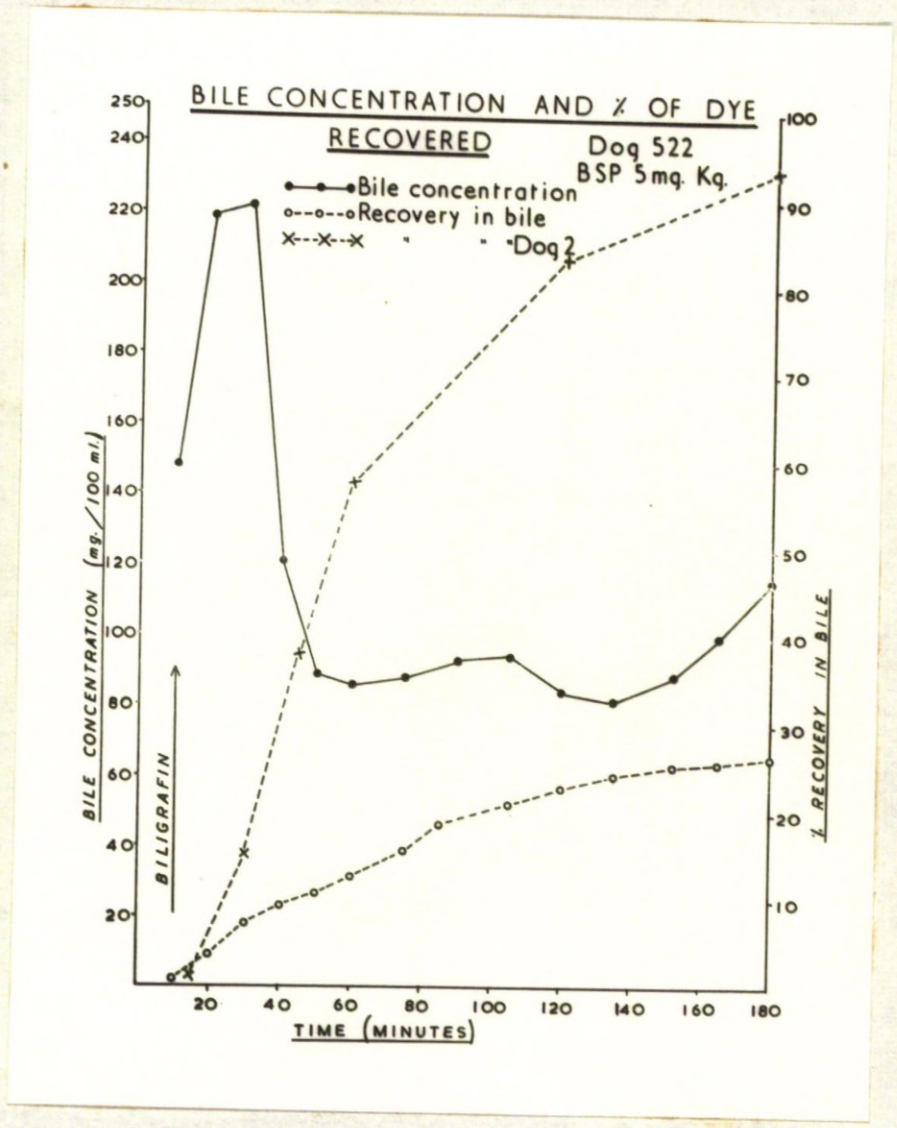
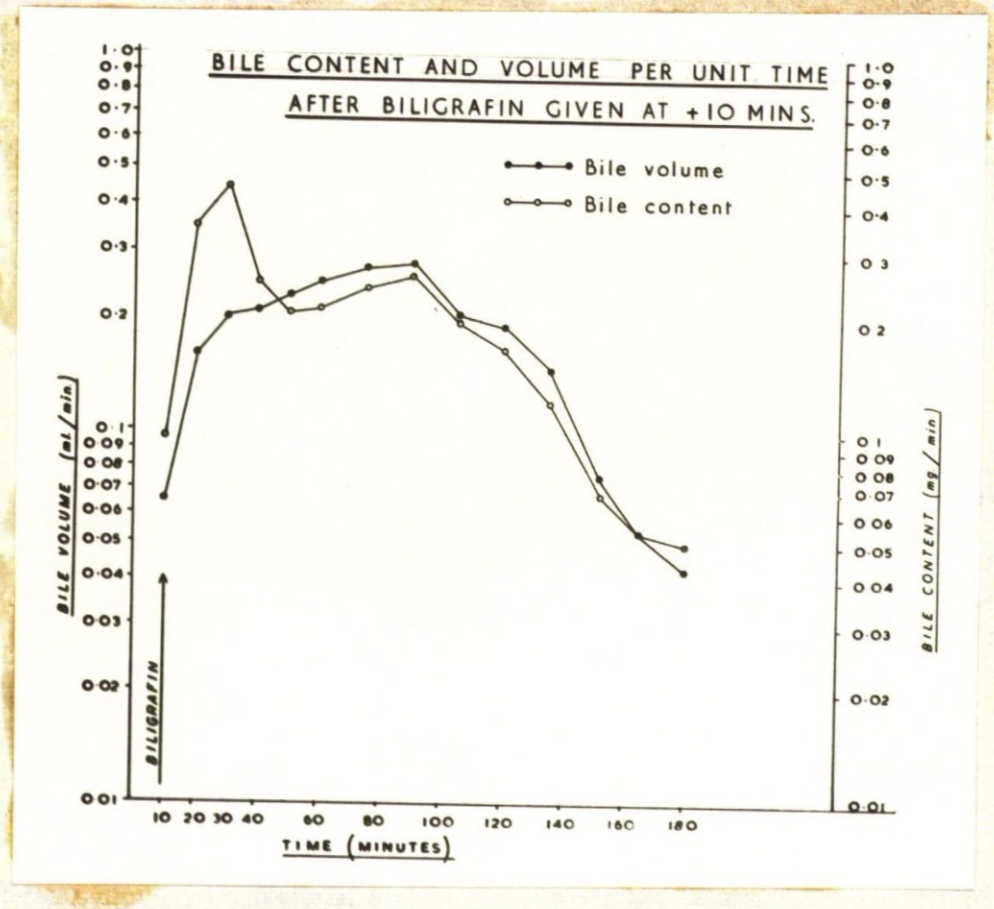

BILE CONCENTRATION AND % OF DYE RECOVERED
Dog 522
BSP 5 mg. Kg.
Bile concentration
Recovery in bile
Dog 2
BILE CONCENTRATION (mg./100 ml.)
BILIGRAFIN
% RECOVERY IN BILE
TIME (MINUTES)
250
240
220
200
180
160
140
120
100
80
60
40
20
100
90
80
70
60
50
40
30
20
10
20
40
60
80
100
120
140
160
180

Figure 101

Biligrafin Series

The bile content and bile volume per unit time following the biligrafin given at 10 minutes. The bile volume in millilitres per minute and the bile content in mg. per minute have been plotted logarithmically against time.


BILE CONTENT AND VOLUME PER UNIT TIME
AFTER BILIGRAFIN GIVEN AT +10 MINS.
Bile volume
Bile content
BILE VOLUME (ml/min)
BILE CONTENT (mg/min)
BILIGRAFIN
TIME (MINUTES)
10 20 30 40 60 80 100 120 140 160 180

acute biliary fistula animals described previously in the Section on the derivation of the mathematical model. It can be seen that whereas 95% of the dose had been recovered at the end of 180 minutes, in that latter experiment only just over 25% of the dye had been recovered following biligrafin.

Figure 101 shows the logarithm of the bile volume (mls./min.) compared with the bile content of bromsulphthalein (mg./min.) in dog 522. The bile volume is increased following biligrafin and this effect is maintained for approximately 90 minutes after which it gradually decreases. The bile content after biligrafin increases rapidly (10 - 30 minutes) to fall to approximately half the value (30 - 50 minutes), after which there is a slight increase in the amount of bromsulphthalein in the bile (50 - 90 minutes) and then a gradual decreasing phase of excretion (90 - 180 minutes).

Summary

The effect of biligrafin, a substance which radiologically is known to be rapidly excreted in the bile, has been given at various times with respect to bromsulphthalein and its effect on the normal dis-

appearance graph for a particular animal observed. When biligrafin was given prior to bromsulphthalein there appeared to be no effect if given 4 hours previously, the suggestion being that either biligrafin is excreted so rapidly, or it is in such a low concentration in the blood and liver at the time bromsulphthalein is given, that it does not exert any effect. A definite effect has been observed when biligrafin was given 2 hours or less prior to the bromsulphthalein, and, on the other hand, the nearer one approaches to a simultaneous injection, the more marked becomes the effect on the disappearance curve. When given simultaneously the effect has been noted to be most marked. The general pattern of behaviour in the doses given would appear to be similar to that of the larger dose of benemid, since with both substances there appears to be (i) an alteration in the initial rapid phase which appears to be delayed; and (ii) affects the second slower phase, which again appears to be altered and proceeds at a slower rate. The actual amount of bromsulphthalein present in the bile under these conditions has, however, not been determined.

A series of experiments in which biligrafin

has been given at various times following bromsulphthalein, confirms that the normal disappearance curves are reproduceable at different time intervals. Following biligrafin, the plasma concentrations are elevated and the rate of disappearance of the dye from the plasma appears to be retarded compared with the expected normal pattern for the particular animal observed. The effects are less marked as one delays the injection of biligrafin, presumably due to the lower concentration of dye in the blood and higher concentration in the liver, but the effect is still present in an animal in which biligrafin was given 33 minutes after bromsulphthalein.

One animal had an acute biliary fistula performed and biligrafin was given 10 minutes after bromsulphthalein. This enabled the bile content of bromsulphthalein to be followed and it has been shown that the recovery of bromsulphthalein in the bile at any stage is less than has been noted in the normal acute biliary fistula dogs. Biligrafin thus alters (i) the uptake and storage of bromsulphthalein by the liver and (ii) the excretion of the dye into the bile. In the doses given this effect appears to be more marked than does the effect of benemid. It

has already been noted that biligrafin also increases the bile volume rate in mls./minute and is again shown in this Section.

XII. DISCUSSION

THE APPLICATION OF THE MATHEMATICAL MODEL TO ASSESS LIVER FUNCTION.

The value of the Bromsulphthalein Liver Function test

In Section II, 2, the functions of the liver are detailed and in the following Section a list of the basic methods of assessing liver function are given. Liver functions can be grouped in many ways and most workers have devised their own groups of tests. Wakim (1954), for instance, described ten groups of processes or functions which were generally accepted as involving the liver in one way or another. Knisely (1951) compiled a list of 84 separate functions which he listed in 18 different ways. Based on the list of functions, Knisely (1951) lists 97 tests of liver function. He was, however, at pains to point out that it was never necessary to carry out all 97 tests to assess liver function and emphasised that none of the individual tests by themselves are sufficient to measure the efficiency of the liver. It is customary to use a small group of tests, usually four or five, in the general assessment of liver function and the selection of these have usually varied, depending upon the preference of the indi-

vidual workers. The literature is full of references as to individual tests or groups of tests, but there is a lack of quantitative studies of the relationship between the different tests. This was rectified in 1955, when a most comprehensive and clear account of relationships of a group of 9 tests was carried out by Zieve and his co-workers (Zieve and Hill, 1955a, 1955b, 1955c, 1955d; Zieve, Hill and Hanson, 1955a, 1955b).

Zieve used the statistical procedures of multiple regression, multiple covariance and discriminatory analysis to take into account and measure the inter-dependents among the 9 tests he had selected. The normal limits of the 9 tests in a group of 720 healthy males was established (Zieve and Hill, 1955b). An attempt was then made to determine the relative effectiveness of each test, considered separately, in distinguishing between an abnormal group and a normal group. In one trial, (Zieve and Hill, 1955c), the abnormals were a group of 41 patients with cirrhosis of the liver and the normal group consisted of 100 healthy males. When a comparison was made between the individual tests it was found that the bromsulphthalein test

(the simple retention test expressed as the percentage of dye retained at 45 minutes following the intravenous injection of a known amount of dye) stood out as the best single test for detecting patients with cirrhosis of the liver, both on the basis of group comparison as well as on the miss classification of extreme individuals. When allowance was made for the inter-dependents among tests, it was found that of the 9 tests, only 4 (the bromsulphthalein test, urine coproporphyrin, zinc turbidity and hippuric acid) had independent value in distinguishing cirrhosis patients from normal patients. Of these 4, only 2 (bromsulphthalein and urine coproporphyrin) had an equivocably significant independent contribution. Zieve and Hill concluded that underlying the results of the separate tests, was a set of common basic factors. Test results which, on the surface, appeared to represent different functional changes, were in fact consequences of a common set of factors. Under these circumstances, Zieve and Hill considered the large battery of tests to be superfluous, since apart from the significant 4 tests, additional tests contributed no new information. With regard to

cirrhosis of the liver, a combination of the four tests was only slightly more effective in discrimination from normal than the bromsulphthalein test alone. This conclusion confirmed the view of Snell and Magath (1938) that in types of liver disease, not associated with jaundice, the information gained from the study of bromsulphthalein retention was as reliable as that which could be gained in any other way and under these conditions other tests gave chiefly confirmatory evidence.

The effectiveness of the group of 9 liver function tests was assessed also in a group of 36 patients with viral hepatitis (Zieve et al, 1955a) The observations by Zieve et al show that the most effective tests of the 9 were the bromsulphthalein retention test and the urine coproporphyrin test, that is, they were the most effective both for group comparison and for the detection of abnormal individuals. When allowances were made for interdependents among the tests, only 4 were found to contribute independently to the discrimination between normal subjects and hepatitis patients. These four were the same four tests which were found most effective in a group of the cirrhosis series (the bromsulph-

thalein test, zinc turbidity test, hippuric acid test and urine coproporphyrin test). It was concluded by Zieve et al (1955b) that the test results observed were the consequences of disturbances in 4 sets of fundamental factors. The same 4 sets of basic factors being involved in cirrhosis of the liver. In cirrhosis, the set of factors accounting for the independent significance of the bromsulphthalein test, were by far of greatest importance. In viral hepatitis on the other hand, the set of factors accounting for the independent significance of the urine coproporphyrin test were most significant. Regardless of any other conclusions made by Zieve and his colleagues it is clear that one general conclusion may be made with regard to the use of bromsulphthalein in assessing liver function. These workers have shown most convincingly in two well-controlled trials, that the simple bromsulphthalein test is both reliable and sensitive in the discrimination of the abnormal from the normal liver in man. That is to say, the bromsulphthalein retention test can be relied upon to select from a mixed group, those cases with either cirrhosis or viral hepatitis. Both would be recorded as "abnormal"

only.

The simple bromsulphthalein retention test cannot distinguish between one form of disordered function and another. It indicates abnormality by the presence of an increased amount of bromsulphthalein in the plasma at a certain time after the injection. It does not indicate the mechanism by which this increased retention comes about. Increased retention could occur in several ways, (i) through the inability of the liver to remove bromsulphthalein from the plasma (ii) through a leakage back to the plasma from the liver cells, the bile or the lymph, (iii) through the inability of the liver to transfer the dye from the liver to the hepatic cells into the bile. Either one or all, or any combination of these disturbances of function could be present and the end result would be the same, that is increased retention of bromsulphthalein in the plasma. Therefore, although the simple bromsulphthalein test can be regarded as reliable in discriminating between the abnormal and normal liver, it cannot, in its present form, be expected to distinguish one form of abnormality from another. For a test to be able to discriminate between one type of disordered

function and another, it is necessary to know the exact relationship between what is measured by the test and the normal 'function' of the liver. With regard to the bromsulphthalein retention test, all that can be concluded when the result is normal is that no more dye, within the accepted normal limits, remains in the plasma at a certain time from the injection. A crude estimate of this kind might well overlook for instance the pattern of dye disappearance from the plasma as suggested by McDonald (1939). Even if the multitude of liver functions be reduced to a few small basic factors, it is quite likely that even the whole battery of tests will fail to reveal a single abnormality, such as the absence of one specific enzyme system, unless there was a particular test which assess this precise and particular function. On the other hand, a specific test would not be a comprehensive test. Therefore, it must be admitted that there can be no single, satisfactory test of liver function, while the possibility remains that there may exist in some diseases an isolated biochemical defect in liver cell metabolism.

The work described in this thesis has been to

introduce a modification of the bromsulphthalein test in order to explain the pattern of dye disappearance from the plasma. Even with the application of this method, it is not suggested that the resulting test will render it the best liver function test, nor will it be a selective specific test, or, indeed, a comprehensive test. It is merely an attempt to extract as much information from a test which has been shown to be one of the most efficient of the liver function tests in present usage.

The four stages involved in the disappearance of bromsulphthalein from the plasma are (i) the passage of the dye from the plasma to the liver, (ii) the passage of the dye, either altered or in its original state, back from the liver to the plasma, (iii) the storage of the dye within the liver cell, either in its original form or in an altered form, and (iv) the passage of dye from within the liver cell out into the bile either in its original form or altered. All these processes could be stated to be functions of the liver and it is possible that these are the basic functions of the liver with regard to any substance which enters the liver cell. An attempt to distinguish between these four pro-

cesses would thus be a good indicator of the whole of liver function. The mathematical model, to a great extent, can be applied to measure these processes. From the mathematical point of view, these have been defined as the rates of transfer of bromsulphthalein between blood, liver and bile and it is suggested that these transfer rates reflect directly the efficiency of the liver cell mechanism and may prove useful in evaluating the whole function of the liver as a whole.

Bromsulphthalein, when used as a substance to test liver function, has been regarded as a substance which is specifically taken up by the liver from the plasma, or a substance which has been predominantly excreted by the liver. Therefore different workers have assessed bromsulphthalein tests as either measuring the efficiency of the liver removing a substance from the plasma or as a measure of the excretory efficiency of the liver. Although variations of the test in common use have suggested that it measured both mechanisms and although experimentally it is widely known that both phenomena are involved in the overall handling in the body, these facts have not been specifically included in any modified test.

Richards, Tindall and Young (1957 and 1959) however, attempted to show that by using the mathematical model it was possible to interpret the disappearance of bromsulphthalein from the plasma in terms of the generally accepted experimental findings, in regard to handling of the bromsulphthalein by the liver. This is the most important physiological factor since the mathematical model is merely an application of similar models which are in common use in several biological fields (Robertson, 1957). Indeed, an almost similar model had been published earlier by Evans, in 1953. The mathematical model proposed by Evans to describe the distribution of bromsulphthalein (after a single intravenous injection) within the plasma, the interstitial fluid and the liver. The expression evolved measured the rate of transfer between these compartments using a data obtained by observation of one compartment only, that is the plasma. The mathematics employed by Evans and the formula he used are almost identical with those which have been evolved by Mr. Andrew Young (see Appendix). The model described by Evans, disregarded all that was known at that time about the distribution of bromsulphthalein in the body. The excretion of

bromsulphthalein into the bile was considered to be unrelated to the passage of dye from the plasma into the liver. Evans' work appears to have been primarily concerned with the 'uptake' of bromsulphthalein by the liver from the plasma. No confirmatory evidence that bromsulphthalein was distributed between the three compartments, the plasma, interstitial fluid and liver, was given. The mathematical model described by Evans (1953) was unable to be adapted to the known physiological facts. That the model which has been suggested by Young correlates with the physiological data is shown in this thesis (Section A of the Results).

In the literature which has been reviewed Sections VII, VIII and IX dealing with liver and bromsulphthalein, it can be accepted that once bromsulphthalein has been injected into the circulation the following physiological processes occur :-
(i) the binding of bromsulphthalein to the albumin fraction of plasma protein, (ii) the transfer of bromsulphthalein from plasma protein to liver protein where the liver cells face the vascular system within the liver, (iii) the transfer of bromsulphthalein from this part of the cell to the portion

of the liver cell which faces the bile duct collecting system, and(iv), the active secretion of bromsulphthalein by the liver into the bile. These are the accepted physiological processes which have been taken into account in the mathematical model but it has also been mentioned in the same Section that there is evidence that bromsulphthalein is not entirely confined to the three compartments, blood, liver and bile. Bromsulphthalein has been noted in the urine and lymph following the single injection of bromsulphthalein. It is also an over-simplification to regard the passage of bromsulphthalein from plasma to bile as involving only the liver cell. The anatomical and physiological evidence which has been reviewed suggests that both the liver, intercellular fluid and liver lymph may be involved at some stage in this process. It is, however, probably incorrect to regard bromsulphthalein in the bile as being finally removed from the inter-compartmental equilibrium between plasma and liver. The bromsulphthalein from bile may return to the plasma by way of the peri-biliary plexus of blood and lymph capillaries, or by virtue of the entero-hepatic circulation by re-absorption from the duodenum.

The mathematical model is based on the fact that a standard injection of bromsulphthalein, 5 mg./Kg. dose is given. As a result of this, the quantities of dye which are concerned in extra-hepatic loss to the urine or interstitial fluid, as well as in the entero-hepatic circulation, are very small compared with the dose of bromsulphthalein which has been given, and the amounts of dye concerned in the blood, liver and bile compartments are very much greater, therefore they are assumed to be negligible in the overall picture. In the 9 animals which were studied, the total amount of dye in the urine during the observed period was never more than 1.5% and in the majority of cases, less than 1% of the injected dose. The two principle assumptions which have to be made physiologically if the mathematical model is to be accepted are that (i) the injected bromsulph-thalein is confined to the compartments, blood, liver and bile, and (ii) with the standard 5 mg./Kg. dose of bromsulphthalein, the extra-hepatic loss and entero-hepatic circulation of dye can be disregarded. Apart from the literature which has been reviewed and shows that the loss of dye under these circumstances is negligible, the experiment detailed for an assess-

ment of the entero-hepatic circulation reveals in fact that the quantities of dye are negligible in that particular experiment which was identical to those performed in the other dogs in this series.

Mr. Andrew Young's mathematical model accepts these two physiological principles and the pattern of transfer of bromsulphthalein between the blood and the liver is accepted, the transfer between them is assumed by the simple laws of diffusion. Mathematically also, the bile compartment is considered to be of an infinite size. Thus, once bromsulphthalein is present in the bile it is accepted that it is incapable of affecting the transfer between the blood and liver. This system can be described as either a two-compartment open system that is a "leak" from one compartment to the exterior, or as a compartment closed system, with the third compartment of infinite size. Mathematically these amount to the same thing and both agree with the physiological assumptions which have been mentioned above. The blood and liver compartments are looked upon as simply two 'volumes' of the solvent, separated by a permeable membrane. A known amount of the "solute" (bromsulphthalein) is accepted as being

injected instantaneously into the plasma. The resulting concentration of bromsulphthalein and the nature of the separating membrane will together determine the quantity of bromsulphthalein transferred in unit time from the blood to the liver. As soon as bromsulphthalein is present in the liver, a random movement of molecules of bromsulphthalein will ensure a transfer of a certain quantity of "solute" of bromsulphthalein back from the liver to the blood. The driving force for the transfer of dye from the blood to the liver is the actual concentration of bromsulphthalein in the circulation, but the actual amount transferred in unit time depends on the concentration (at any time) in both the blood and liver compartments (i.e. the net transfer between the compartments depends on the concentration difference between the two compartments). The situation thus depicted can be presented in a form which enables the basis of the mathematical model to be understood. If x is equal to the concentration of bromsulphthalein in the blood at any time, t, and y is the concentration of bromsulphthalein in the liver any time, t, and 'a' is the constant proportion of bromsulphthalein molecules which are transferred from blood to liver and 'b' is

a constant proportion of bromsulphthalein molecules transferred from the liver to the blood, then $\frac{dx}{dt} = -(ax + by)$, i.e. the rate of change of concentration of bromsulphthalein in blood is equal to the amount leaving the blood (- ax) plus the amount entering the compartment (+ by) in unit time. The overall change in concentration in unit time, therefore, depends on both x and y (a concentration of bromsulphthalein in the plasma and a concentration of bromsulphthalein in the liver). If the liver has a 'leak' to the bile, mathematically assumed as a compartment of infinite size, or a loss to the exterior is considered, then the following equations will be derived if 'h' is equal to the constant proportion of bromsulphthalein molecules transferred from the liver to the bile, then $\frac{dy}{dt} = ax - (b - h)y$ and if z is the concentration of bromsulphthalein in bile then $\frac{dz}{dy} = hy$.

In the mathematical appendix the solution of these three differential equations is given and has the form C_{x_t} is equal to $Ae^{-k_1 t} + Be^{-k_2 t}$. C_{x_t} is equal to the concentration of bromsulphthalein in the blood at any time, t, and A, B, k_1, k_2 are constants. In the Section dealing with the temporal

distribution of bromsulphthalein in the body, it has been shown graphically in Figure 32 that the semi-logarithmic plotting of the observed bromsulphthalein concentration against time can be used to obtain the values of the constants A, B, k_1 and k_2. It was also shown in this Section as well as in the mathematical appendix how, once these constants have been determined, it is possible to obtain values for the constants of proportionality a, b and h. Once these constants have been obtained, it is possible to calculate at any time, t, the bromsulphthalein content of the plasma, bile and liver (Richards, Tindall and Young, 1959).

The mathematical model regards the blood and liver as a fixed volume of solvent in a given animal and uses the simple diffusion method of transfer of dye. It is obvious that neither the blood nor the liver can physiologically be regarded as simple solvents, and as has been mentioned in the Sections dealing with a review of the experimental findings of "The Cell and Secretion" and "Dyes and the Liver" that, with regard to bromsulphthalein, processes other than diffusion are necessary to explain the experimental findings. Despite these obvious differences between

the mathematical assumptions and the physiological facts, there are two main reasons for considering that the suggested model is relevant. Firstly, because both the blood and the liver contain protein (plasma and liver albumin). Bromsulphthalein has been demonstrated to be bound to albumin in the plasma and will presumably become bound to liver albumin, once it enters the liver cell. It has been definitely shown that it combines with liver protein in vitrol and did in the experiments on dog liver, but not in vivo. Therefore it can be considered valid that bromsulphthalein is distributed following its injection into the circulation between two sets of protein, one in the liver and one in the blood. The effective concentrations of protein will, therefore, determine to a large extent the concentrations of bromsulphthalein in the blood and the liver. The quantities of protein in the blood and liver are regarded as "volumes of solvent" for the purposes of the mathematical model. Secondly, the mathematical model, although based on a system where a simple diffusion is the only mechanism of transfer, should only be regarded as describing the end results of a series or chain of processes. The actual amount of

dye transferred from one compartment to another depends on the concentration gradient of the dye and on the nature of the membrane separating the blood and liver protein. The single membrane of the mathematical model is equivalent to the physiological functioning liver cell.

One of the functions of the liver cell is active secretion and since it can actively secrete, it is only able in unit time to secrete the amount of bromsulphthalein presented to the secretory mechanisms of the cell. The amount presented depends on the concentration of bromsulphthalein in the cell, and ultimately in the blood. Thus the end result both in the mathematical model and the liver cell is the same, that is, that the concentration of bromsulphthalein within the cell will govern the amount of bromsulphthalein excreted to the bile in unit time.

It is not suggested that the mathematical model derived by Mr. Andrew Young will be able to prove, or disprove, any of the accepted ideas of bromsulphthalein distribution within the body. The model is only an attempt to express in quantitative terms the distribution of the dye and a

model such as this can only be accepted as useful if it agrees with the experimental observations. It is suggested that the results on nine dogs in Section A of the results provide fair evidence for the physiological acceptance of the mathematical model.

It is impossible to confirm or dispute the values for the transfer constants since these cannot be estimated by any other means than the mathematical model. There can be no direct independent method of assessing their accuracy. The model calculates the values for the transfer constants between the blood, liver and bile and, using these, the actual quantity of bromsulphthalein in each of the three compartments can, at any time, be determined, following the injection of bromsulphthalein. It is, however, possible to compare the quantities of bromsulphthalein in the compartments with those calculated by the mathematical model. Some of these can be compared at any given time but others can only be determined as a terminal event.

It is accepted that, and it is important to realise, that on no occasion was a complete agreement found between the 'observed' and 'calculated'

quantity of bromsulphthalein for the three compartments and I must give a few suggestions to account for these differences. The value for the plasma volume was regrettably not determined in any of the animal species used, but since we are considering the mathematical model the only animal initially concerned is the dog. A value taken for plasma volume was based on the body weight. Whatever value is taken for the plasma volume on a body weight basis will, in an obese animal, tend to over-estimate the plasma volume, whereas in the thin, leaner type of animal, the plasma volume will be often under-estimated. A comparison of the values has been given, using either 50 mls./Kg. body weight or 53.9 mls./Kg. body weight, the latter being the average of 40 normal mongrel dogs, obtained by Gibson and his colleagues (1946). However, if the plasma volume in a particular dog is taken to be that indicated by the volume of fluid in which the bromsulphthalein was present, that is in the zero concentration value, then a closer agreement between the results would be obtained. The rapidity of the loss of bromsulphthalein from the circulation, however, renders it an unsuitable substance for the estimation of plasma volume, but the mathematical model has to

accept the volume in which bromsulphthalein is present at zero time as the plasma volume. Therefore, if the observed and calculated plasma concentrations were multiplied by the volume given by dividing the dose of bromsulphthalein given by the zero concentration obtained by interpolating the observed concentrations back to zero time and multiplied by 100, it would give the bromsulphthalein compartment space and then the conversion would yield plasma quantities and the agreement would then be very close.

The amount of bromsulphthalein which has been recovered from the bile has always been less than the amount predicted to be present. There are two main reasons for this :- (i) the inevitable dead-space delay and (ii) the failure of the colorimetric method to detect the total amount of bromsulphthalein present. The dead-space in the biliary tree and cannula is unavoidable since bile is formed by the liver cells at time t, and is not available for collection and estimation until a time t - D, where D is the time (in minutes) taken for the bile to travel from the site of formation to the site of collection. When allowance is made for some of the time dead-space, then the observed and calculated quantity of bromsulph-

thalein agree more closely. It is difficult to determine exactly what the time delay in a particular experiment will be for the dead-space. It has already been pointed out in Section VII, dealing with bromsulphthalein and its distribution in the body, that the colorimetric method used for estimation of bromsulphthalein in the bile gives values which fall short of the values given by radio-active methods of estimating the bromsulphthalein. The quantities of dye recovered in the bile, however, in this series of experiments, appear to have yielded a consistently higher proportion of dye than the corresponding colorimetric method of Brauer and his colleagues in 1955. The overall picture if allowance is made for both these factors shows that the observed calculated quantities of bromsulphthalein in bile is reasonably good.

As has been mentioned previously, it is not possible to compare the liver content directly at any individual time during the experiments which have been given in Section A of the Results. It is therefore impossible to obtain a direct, continuous check on the calculated values of dye present in the liver. The liver content of dye at the end of each experiment

was determined and compared with the calculated content at that time. The method used for determining the content of bromsulphthalein in the liver unavoidably results in the denaturation of liver protein. Since the majority of bromsulphthalein in the liver can be expected to be bound to protein, only a certain proportion of dye will be available for recovery, using this method. Experiments on cat liver appeared to yield a consistent recovery range, whereas the recovery from dog liver was not so precise and indeed only yielded on an average a 30 - 40% recovery. The differences may well have been due to the different diets of these animals and/or possibly the different type of protein material present. The results which have been given in Section A show that the range of the expected content of bromsulphthalein corresponds reasonably with the calculated quantity. Another reason for accepting the pattern of hepatic uptake of bromsulphthalein, as described by the mathematical model, is the fact that it appears to be almost identical with the pattern which has been described in rabbits and human subjects by Taplin, Meredith and Kade (1955) for the hepatic uptake of radio-active Rose Bengal.

If one is prepared to accept the limitations necessarily imposed by the inadequacy of the methods used for checking on the predicted distribution of bromsulphthalein by the mathematical model, then the mathematical model of bromsulphthalein distribution within the body is in accord with the experimental findings. Accepting this, it seems valid to conclude that the transfer constants, or rates of proportionality for the transfer of blood to the liver, the liver to the blood and the liver to the bile ('a', 'b' and 'h' respectively) have a physiological meaning which can be used as an indication of the level of hepatic cell efficiency. The main animal which has been used throughout this work has been the dog and the mathematical model has been based on the findings in a series of nine dogs. In order to see if this method could be applied to other animal species, serial estimations of the plasma concentrations of dye following the injection of a standard dose of bromsulphthalein have been tried. In certain animals, the liver appears to be relatively more efficient in its ability to take up the dye from the blood so that the initial rapid phase takes place remarkably quickly and there is difficulty in obtaining a sufficient

number of observations during this phase. It has been shown in hens, rats, sheep, cows, goats and even the horse that there is a rapid disappearance of the dye, so that the blood levels of the bromsulphthalein are approaching the outside lower limits of accuracy of the colorimetric estimation methods, within 15 - 20 minutes. In some of the animals, cats for example, there is the impression that as this lower limit is being approached, so the "bend" of the second slower phase of the double, exponential pattern is being reached and that the semi-logarithmic straight line representation is due to the recordings of the observed concentrations during the initial rapid phase. The possible explanations of the different types of graphs obtained when the results are plotted logarithmically, have been given in Figures 70 - 73. Another possibility, as explained above, is that the relative amounts of protein within the two compartments, blood and liver, are the explanation of the different results in a particular species. Heamatocrit readings of the proportion of cells to plasma vary considerably in different animal species and as a result the plasma volumes are also variable and similarly total protein content.

The actual amounts of protein in the two compartments, blood and liver, may only reflect the disappearance pattern since if there is a relatively large amount of liver protein available for bromsulphthalein, then with the generous circulatory pathway through the liver substance, a greater proportion of dye may be extracted in any particular passage of dye through the liver. The ability of the liver to excrete bromsulphthalein into a third compartment, the bile, may be a significant factor, since the more rapid the removal of dye from the liver to the bile, the greater will be the number of available sites for attachment of bromsulphthalein at any given time. It is a pity that in each animal species investigated the plasma proteins and the amount of liver protein was not determined, since this may well have helped to indicate whether or not the absolute quantities of protein present is a significant factor. With the limited ability of the liver to excrete certain substances in the bile, there is a limit to the amount of bromsulphthalein which can be taken up by the liver, but these conditions are unlikely to be attained following a single intravenous injection. Even in the cat, which received a very large dose,

of the order of 63 mg./Kg. body weight, the liver appeared to be able to excrete the dye satisfactorily, judging by the rate of fall of the observed plasma concentrations in this case. In one dog, which was given a relatively large dose, 20 mg./Kg. body weight, there was a phase in which there appeared to be a steady plasma level and it was considered possible that saturation may have occurred. This, on reflection, however, is unlikely, since other dogs given equivalent quantities of dye have not yielded plasma concentrations which follow the same pattern. In the only experiment in which the common bile duct was obstructed (Table 14) there followed a phase where dye disappeared from the plasma, presumably due to its being taken up by the liver and following this there was a steady plasma level which appeared to be gradually rising and then Sodium Dehydrocholate was given on two separate occasions and, following each injection, a raised plasma level of bromsulphthalein was obtained. It is, of course, always difficult to interpret experiments in which the common bile duct is obstructed, since back-pressure may damage the liver cell, with the result that a certain amount of bromsulphthalein would be returned into the circulation.

Table 14

The Effect of Obstruction of the Common Bile Duct.

In this 10 Kg. dog, the common bile duct was cannulated and then obstructed to a pressure of approximately 16 - 20 mgs. of mercury. A dose of bromsulphthalein of 75 mg. (7.5 mg./Kg.) was given at zero time. The plasma concentration falls in the first twelve minutes, a fall of 75% of the three minute plasma concentration, but after this it maintains a steady and gradually increasing level until Sodium Dehydrocholate is given at 81 minutes, when the plasma level concentration increases once more. When a further injection of Dehydrocholin is given, then the elevated plasma concentration is still maintained during the period of observation.

Dog Weight 10 Kgs.

75 mgms. of Bromsulphthalein given.

The cystic duct was clamped and the common bile duct cannulated. The common bile duct was then obstructed to a pressure of 16 - 20 mgms. of mercury and Sodium Dehydrocholate was given (at 81 minutes and 112 minutes). The plasma concentration of Bromsulphthalein (mgms./100 mls.) is shown at the respective times.

Time in Minutes	Mgs/100 mls.
3	8.8
6	4.41
9	2.83
12	2.18
15	2.19
21	2.78
30	2.28
40	2.78
50	3.10
60	3.06
78	2.86
83	3.22
86	3.60
90	3.88
102	4.07
110	4.22
114	4.00
120	4.25

Urine content of Bromsulphthalein 1.45 mgms.

The Bromsulphthalein Disappearance Curve

Regardless of the significance of the disappearance curve mathematically and its interpretations, it has been shown that the bromsulphthalein disappearance curve in a particular animal is reproduceable at different times and that there is a normal range for a given species of animal. The effect of operations have been to elevate the observed plasma concentrations, although this appears more particularly with the performance of an acute biliary fistula. An ordinary laparotomy did not appear to alter the normal disappearance pattern. The importance of the observations of Tagnon, Robins and Nichols (1948) who showed that there was an increase in the retention of bromsulphthalein immediately after an extra-abdominal operation in human subjects, lies in the fact that the immediate surgical trauma that affects the disappearance curve may be directly but partly due to the circulatory effects on the body as a whole. It has been found important, therefore, to allow a reasonable control period before observing any effect on the bromsulphthalein disappearance curve. Dog 10 shows the effects in an animal who was not allowed a control

period and had not, in fact, recovered from the surgical trauma associated with the operation, as well as failure to maintain the body temperature at its normal level. The importance of anaesthesia has not been determined here since all the experiments on dogs were performed under anaesthesia and the experiments on hens, sheep, goats, cows, horse and human subjects, were in the none anaesthetised state.

The removal of bromsulphthalein from the circulation depends upon the ability of hepatic cells to take up the dye from the plasma and partly on the blood supply to the liver. In many diseases in which plasma retention of bromsulphthalein has been noted to be high, there is an associated abnormality of the liver cells. Popper and Schaffner (1957) give cirrhosis, hepatitis, cholestasis, carcinoma and a fatty liver as the hepatic diseases which are associated with bromsulphthalein retention. The extra-hepatic conditions given by these authors to be associated with bromsulphthalein retention are congestive heart failure, fever, malaria, infections, shock, gall-bladder disease and surgery. The importance of the hepatic circulation in the

evaluation of the bromsulphthalein test in this second group probably explains the abnormal results in congestive heart failure or shock without any apparent damage of the hepatic cells and the high retention which occurs when slight damage of the liver cells is associated with circulatory defects. The interference with the portal venous blood supply into the liver has been shown in the Results in Section B. The immediate effects of acute exclusion or a by-pass operation are dramatic. The effects of a gradual reduction of the blood supply to the liver by virtue of a portal vein tie, as a two-stage operation, has also been studied and the bromsulphthalein disappearance curve is altered. One explanation may be due to the reduced liver weight which occurs in these animals and hence an anticipated reduction in the amount of liver protein, the proportion of blood protein to liver protein is altered and therefore the 'abnormal type' of graph occurs, providing the dog has maintained its weight, although following this particular operation this does not always occur.

It has been reported that a bromsulphthalein test is abnormal at the time of menstruation. This

might possibly be due merely to the increased body temperature or possibly to the hormonal changes associated with this state. Certainly in the dog which was noted to be on heat there was an elevated plasma concentration at all times. It has been reported by Campbell (1957) that the bromsulphthalein disappearance curve in hens is different from that in cocks and that it is possible to change the 'male type' curve to 'female type' by the administration of oestrogens to the cocks. In this fowl series much larger doses of bromsulphthalein were given than to the dogs presented in this work, where it has been impossible to find any significant difference between the bromsulphthalein disappearance graphs obtained from either sex. In fact, the sex has not been specifically mentioned in the results because there was no apparent difference.

The effect of the dose of bromsulphthalein appears merely to increase the observed plasma concentrations. Mathematically, the behaviour is similar and the rates of proportionality can be shown to be the same in any given case, it is merely that one is dealing with a different load in the respective directions due to the increased

quantities of dye present. A slight difference in the observed graphical results is due to the relatively few number of observed concentrations which have been determined during the rapid initial phase. The result of repeated injections of bromsulphthalein at short intervals of time reveals different disappearance graphs, but as explained in the mathematical appendix, the behaviour pattern of the liver is not altered, the graphical results merely being a reflection of the inadequate number of samples taken during the respective phases of transfer.

The Effect on Bile Volume

It has been noted during the course of this work that many of the substances including bromsulphthalein itself, a finding which has not been reported generally, have a definite choleretic effect. It has been impossible to estimate the relative effects of the various substances separately which have been used in competition with bromsulphthalein directly. The increase in flow seems to be partly determined by the dose of the substances used and partly by the pre-existing or control rate of bile volume flow. The ability to increase bile volume flow is a property shared by a great many substances, apparently

unrelated to each other. The literature describing their types and structure is quite voluminous. The bile acids (the taurine and glycine conjugates of 3 - 7 - 12 trihydroxycholanic acid) are regarded as classical examples of cholagogues, that is, substances which increase bile volume flow, and they have been frequently used as a yard-stick with which to measure the effectiveness of other, usually synthetic cholagogues (Berman, Snapp and Ivy, 1940). Secretin is another substance which has been noted to promote the formation of bile in man (Grossman, Ganowitz, Ralston and Kim, 1949) and in dogs (Tanturi, Ivy and Greegard, 1937). Although the bile acids and secretin and many other substances increase the volume of bile produced in unit time, they do not all have the same effect on the constituents of bile. Berman et al (1940), Brauer and Pessotti (1952) found that the conjugated bile acids not only increased bile flow but also increased the solid content of bile, whereas the oxidised unconjugated bile assets increased the flow of bile with constant or diminished solid content. The first group were called 'choleretic' substances, that is, the bile produced after administration of the substance was much the same as

the bile produced beforehand. The second group of substances were called hydrocholeretics, that is, the bile produced after the administration was equivalent to control bile to which water had been added. In general, it would seem that all the synthetic cholagogues so far studied are in fact hydrocholeretic substances. Cook, Bianchi, Hambourger and Green (1950), Cook, Lawler and Green (1954), Gunter, Kim, Magee, Ralston, Ivy (1950), Magee, Kim and Ivy (1952). These and other workers have, however, failed to correlate any structural relationship between the substance examined and its hydrocholeretic effect.

All the substances which have been used in this study appear to be hydrocholeretic agents, with the exception of Sodium Taurocholate. Hober (1940) has suggested that all substances which increase the water of bile are surface active substances but the method of their action has not been explained. Bizard and Vanlerenberghe (1956), despite a detailed study of the phenomena have been unable to give any definite answer on this problem. It has been discussed earlier in a review of the literature that there is good evidence to believe that water is actively secreted by the liver cell under normal

conditions and therefore it is reasonable to accept that alteration in the water content of bile must be related to an alteration in the active secretory process rather than to changes in passive infiltration from plasma to bile. The action of choleretic or hydrocholeretic substances would appear to involve basic liver cell processes and their administration disturbs the normal intracellular process of transfer and secretion. The result is that certain substances, for example, bromsulphthalein and bilirubin, are excreted at a slower rate and others, such as water and electrolytes are excreted at a faster rate. According to Goetzee, Richards and Tindall (1960), benemid in the dog increases the water content of bile and decreases the rate of excretion of bromsulphthalein, but it does not appear to alter the quantity of bilirubin excreted in unit time. It was therefore concluded that benemid disturbs normal intracellular transfer processes and renders the liver cell less efficient in its handling of injected bromsulphthalein. Biligrafin, which has also been used in this work, appears to be a hydrocholeretic agent acting in a similar manner to benemid, although it has been

impossible to find an easy method to determine the amount of biligrafin in the bile. The subsequent pattern of bromsulphthalein removal from the blood following biligrafin in the dosage used, appears to follow a similar pattern to that of the larger doses of benemid and similarly, Sodium Dehydrocholate, a potent hydrocholeretic substance, behaves in a similar manner. Sodium Taurocholate, on the other hand, does not appear to affect the excretion of bromsulphthalein as the other substances. No mention of the bile volume can be given without reference to the specific effect of bromsulphthalein itself, as a choleretic agent. This has been a consistent finding throughout this work, although Bizard and Vanlerenberghe (1956) have stated that bromsulphthalein has no such effect. The only reference to an increase in bile volume after bromsulphthalein has been that of Sperber (1957) in chickens.

The group of experiments on bile volume, as well as the other experiments carried out throughout this work are conclusive proof that excretion of bromsulphthalein is accompanied by an increase in bile volume flow. The magnitude of proportional increase would appear to depend on the pre-existing

control bile flow rate and this, in turn, depends on the rate of excretion of both newly-formed and re-circulating bile salts. The mechanism for the increase in bile water which accompanies bromsulphthalein excretion, is probably similar to that of the choleresis produced by bile salts, benemid and biligrafin. Bile salts pass from the liver cell into the bile and provoke an increase in water excretion at the same time. It has been suggested that benemid follows a similar pattern and it is likely that bromsulphthalein and biligrafin do the same. All these substances then share other properties with bile salts, that is to say, in the process of transfer across liver cells of bromsulphthalein, it becomes bound not only to carrier proteins but to specific proteins of the phosphatase enzyme systems and so reduces the amount of energy available for certain specific cell processes. Therefore, bromsulphthalein, in the process of being excreted itself, modifies the behaviour or efficiency of the liver cell. If we can use the effect of choleresis as a guide, then it would appear that bromsulphthalein in the doses given, is the least likely of all the substances that have been used in this work to upset

liver cell function, since the doses of Sodium Dehydrocholate, benemid and biligrafin used have produced a much greater choleretic effect. The effect obviously appears to be related to the dose, since when benemid is given in small doses, it hardly affects the proportionality rates of transfer, 'a', 'b' and 'h', whereas the large doses of benemid, Sodium Dehydrocholate and biligrafin, generally speaking used in this work, have had a profound effect. Also, it cannot be denied that bromsulphthalein itself must in some way modify the cell and the membranes through which it passes during the process of excretion, but its effect is slight compared with the marked effect of the other substances.

It is difficult to be certain that the effects of these hydrocholeretic substances, which have been used, are not due to an effect primarily on the blood flow, since the rate of transfer of dye from the plasma to the blood depends on the rate of volume flow through the liver as a whole. This constant of transfer 'a' is (i) really an indicator of the efficiency of the liver cells to take up or extract dye from the plasma (ii) the rate of the volume flow of blood through the liver, and (iii) the number of channels which are

available for the blood to circulate through the liver at any time, since not all sinusoids are open at any given time. Thus, this rate of transfer would be markedly affected by an alteration in blood flow, whereas the other constants of transfer from the liver to the blood, 'b', and liver to the bile, 'h', are unlikely to be affected by blood flow, since they measure the ability of the liver cell to deal with a certain quantity of bromsulphthalein within the cell and do not reflect any change in the rate at which a substance is presented to the cell initially. Therefore, the fact that in the benemid series all rates 'a", 'b' and 'h' are affected, means that the blood flow is unlikely to be particularly affected. Similar results have been obtained in the biligrafin series where the rates of transfer, 'a', 'b' and 'h' are all altered, when given for up to two hours prior to the injection of bromsulphthalein. According to Baer (1950) benemid has not been demonstrated to affect renal blood flow despite it affecting many phases of renal secretion.

The substances which have been used to judge their effect on the bromsulphthalein disappearance

curve obviously have some competitive effect for liver protein but it is considered that, because of their necessary effect on bile volume, they must have a direct action on the cell processes within the liver cell. The resulting effects on the bromsulphthalein disappearance curve will be partly due to direct competition for liver protein but, in the main, will be due to the actual interference with the liver cell processes.

A hypothesis to explain disturbed liver function in terms of the mathematical constants, when bromsulphthalein is challenged with other substances.

One of the main purposes of examining the effects of various substances on the bromsulphthalein disappearance curve has been to see if they might similate conditions of liver damage or impairment. It was thought that if measurable degrees of "damage" could be inflicted on the liver, then if the mathematical model test of function is used, it ought to reveal the degree of damage in quantitative terms. It has to be decided whether or not the alteration in the handling of bromsulphthalein is due to liver damage, or whether it is due simply to competition between the two substances. Competition obviously plays some part in the process, but it is difficult to be certain of its exact role.

The distribution of bromsulphthalein between the plasma and liver, following a single injection of the dye can be described in terms of 'bound' and 'free' dye. The liver cells are presented in the first place with plasma containing free bromsulphthalein and bromsulphthalein bound to plasma albumin; 'free' bromsulphthalein is thus available for binding

with the protein of the surface of the liver cell exposed to the blood stream and so some of it will become bound to any available liver protein. Thus, 'bound' and 'free' dye exist in equilibrium with each other in the blood and removal of dye by binding to liver protein of 'free' bromsulphthalein will immediately produce a new distribution of dye in the plasma. Equilibrium between 'bound' and 'free' dye will be restored by a decrease in the amount of bromsulphthalein bound to protein. More free dye will then be available for binding to liver protein, and again, some will be removed from the blood by such binding. If there were no other factors operating, this process of transfer of bromsulphthalein from plasma albumin to liver protein would continue, until equilibrium was reached between the amount of dye bound to plasma and in the liver. However, at least two other factors must be taken into consideration (i) it is evident that of the total amount of free dye available to the liver cell protein for binding, some is not so bound but passes into the cell by simple diffusion, according to Hober (1945), and (ii) the entry of bromsulphthalein into the cell is not restricted to simple diffusion

but can occur by contraction of bromsulphthalein bearing protein molecules present in the cell surface (Goldacre and Lorch, 1950; Goldacre, 1952). The operation of both these factors ensures the passage of bromsulphthalein from the cell surface to the cell interior and so prevents equilibrium conditions arising between the plasma and the liver cell surface facing the blood stream.

Bromsulphthalein, once it has entered the liver cell, will follow a pattern similar to that which occurs in blood. Some bromsulphthalein will be 'bound' to intercellular protein and some will be in the 'free' state. Of the bromsulphthalein bound to intercellular protein, some will be bound to protein structures forming an essential part of the transferring of secreting function of the cell. These structures have been called 'carrier' proteins by Hober, (1945). The function of these 'carriers' would seem to be the transport of bromsulphthalein from the blood (plasma) face of the liver cell to the excretory mechanism of the liver cell surface adjacent to the biliary tree. The total amount of bromsulphthalein passing across the cell is therefore composed of two functions (i) the result of

diffusion and (ii) the result of active transfer by means of intracellular carriers. The process of excretion of bromsulphthalein from the liver cell into the bile seems to consist of the active removal of bromsulphthalein from liver cell protein, since bile contains only trace amounts of protein and yet can exhibit concentrations of bromsulphthalein many times, 100 - 1,000, greater than those existing in the plasma.

This hypothetical carrier system requires a source of energy for the work done in moving the carriers across the cell and for setting free bromsulphthalein from its attachment, for its elimination from the cell. Danielli (1952) has suggested that the phosphatase enzyme systems are the source of energy for the contraction of surface protein changes, and other processes which are involved in secretion. Further, it may be pointed out, that the direction or shift of bromsulphthalein from the surface of the liver cell facing the plasma to the surface of the liver cell facing the biliary tree, is probably determined by structural components of the filamentous mitochondria which provide intracellular channels parallel to the long axis of the cell (see the Section

on the Liver Cell).

The process of uptake, storage and excretion of bromsulphthalein by the liver, which involves protein binding and the availability of energy for active transfer, has been shown in the experiments described above, to be disturbed by the administration of some other substances. Of the substances which have been used, Sodium Taurocholate, Sodium Dehydrocholate, benemid and biligrafin have been shown to affect all phases of dye uptake and removal. Of these four substances, it is known that benemid is absorbed on to protein (Tillison, Schardt, Fishman and Beyer, 1954) and that some of the effects of these substances must be by competitive absorption for protein sites within the liver cell. When these substances are given following bromsulphthalein there is a reflux of dye into the plasma. Under these conditions, the proportion of 'free' dye in the liver and in the blood would be raised, but this, in itself, does not explain the changes seen in the rate of transfer of dye, 'a', 'b' and 'h'. The amount of bromsulphthalein transferred from plasma to bile depends not only on the concentration of 'free' dye but also on the number of

'carrier' sites available. The first is normally a proportion of the total dye present and the second may be regarded as a normal function of the cells. The quantity of dye transferred would thus be a function of the produce (concentration of free dye) times the number of sites available for transfer and excretion. In considering the effect of substances on such a transfer system and the effect on bromsulphthalein, it is necessary to assess the part played by two factors. One, the relatively and absolutely larger quantities of free bromsulphthalein presenting for excretion as a consequence of the action of the substances in the liver cell, and, two, the reduction of such secretory sites made by the competitive absorption of substances upon them. The number of protein sites available for transfer or excretion of bromsulphthalein and also the amount of energy available for such work to be done are important. Benemid has been shown to reduce the availability of such energy by Beyer (1950, 1954) in experiments where the renal elimination of various substances was inhibited by benemid. Benemid then, not only competes with bromsulphthalein for absorption on to carrier and excretory sites

but it is also absorbed on to the protein of the phosphatase enzyme systems and so reduces the amount of energy available for cell work. The general trend of these two factors is in opposite directions, the first tending towards increased transfer of dye and the second to decreased transfer and the observed result will obviously be a balance of the two.

The other substances which have been used to compete with bromsulphthalein excretion have not been so thoroughly investigated for the presence of binding with protein. It would appear, however, that both Sodium Dehydrocholate and biligrafin, being hydrocholeretic substances, may well act in the same manner as benemid. Sodium Taurocholate, which is a known choleretic agent, has a different effect. It has been noted, for instance, that the recovery of bromsulphthalein does not appear to be, in fact, reduced following Sodium Taurocholate, which is known to increase the normal constituents of bile. With the hydrocholeretic substances, in which there is a large increase in the water and electrolyte content of bile, there is an associated decrease in the amount of bromsulphthalein transferred. Even when allowance is made for the volume and the amount of

bromsulphthalein present, the overall excretion of bromsulphthalein is reduced. This has been depicted in several of the Figures depicting experiments in which acute biliary fistulae have been performed. The amount of dye recovered under these circumstances in the bile has been even less than in an animal who was suffering from an elevation of the body temperature and had not fully recovered from the operative effects of surgical trauma. Although the results have not been submitted to the mathematical treatment through an electronic computer, the constants, 'a', 'b' and 'h' following benemid and biligrafin, as well as Sodium Dehydrocholate, can all be shown to be raised when treated graphically. As a result, it would appear that these substances in the doses given, cause some degree of liver impairment and it is an interesting point to note that the dose of the substance used appears to be important. In the few cases in which a small dose of benemid was given, there was noted to be an increase in the rate of bromsulphthalein excretion, but when larger doses were used, there was a distinct fall in the rate of excretion of bromsulphthalein.

It has been shown that the simplified method of

obtaining the results graphically compared with the more complex calculated results might well be suitable for clinical purposes. The errors which might attain in estimating the initial rapid phase graphically, may be of the order of 10%, which is considered too large mathematically, and minor degrees of alteration may not be considered mathematically significant. The results following biligrafin, benemid and Sodium Dehydrocholate in this work have given alterations in the slopes of the initial rapid and second slower phase of the graphical results of a much greater order than 10%, so that is is reasonable to consider the results significant. In the final analysis, however, these results should be treated by processing through an electronic computer to be certain of the significance of the results, but, unfortunately, this has not been possible.

The overall impression is that these substances are capable of inducing some degree of liver 'damage' and under these conditions there appears to be a consistent alteration of the rates of proportionality for the transfer of dye given by the mathematical model. It therefore seems reasonable to anticipate that the model will prove useful as a

clinical application.

In all the animal species investigated, there is confirmation that serial estimations of a plasma concentration yield more information than a single, solitary specimen at any given time. In the group of sheep in which liver damage was induced, the alteration in the disappearance curve is quite marked. Subsequent work which has been carried out by my former colleagues, Goetzee, Richards and, a new member to the team, Mr. Thompson, a mathematician, has shown that benemid induced reversible liver damage and that the mathematical model is capable of detecting minor degrees of liver damage when the observed results are programmed in the electronic computer and showed that the mathematical constants have yielded satisfactory results giving evidence of liver damage. They have also carried out experiments on human volunteers (students) and have tested the results (obtained initially in dogs) on the human subject, where benemid has again been shown to give a transient impairment of liver function, or, perhaps it is better to say disturbed liver cell function, for a short period of time. The mathematical model is now being used on clinical cases of liver damage and apparently

it is giving a quantitative assessment of the processes affected.

XIII. SUMMARY

Summary

The disappearance of bromsulphthalein from the plasma as a serial estimation has been carried out in various animal species. The results obtained have been shown, when plotted as the semi-logarithmic plasma concentration against time, to reveal a double exponential graph or a single exponential type of graph. The reasons for the apparent differences in the animal species investigated, have been debated. In individual species, however, it has been shown that impairment of the liver can be revealed by an alteration in the normal disappearance graph.

The distribution of bromsulphthalein has been studied in cats and dogs, but especially in the dog, where a derived mathematical model to explain the temporal distribution of the dye following a single standard intravenous injection of 5 mg./Kg. body weight, has been compared with the experimental findings. Derivation of this model assumes that under the conditions of the experiment there is negligible loss of dye to any compartment other than the blood, liver or bile.

The effect of operations, interference

with the portal venous circulation, the effect of dose and repeated doses of bromsulphthalein have also been studied on the disappearance graph obtained in dogs and the significance of these results has been discussed.

The direct effect of bromsulphthalein on bile volume has been studied. Various other substances which are known to be choleretic or hydrocholeretic agents have also been studied, including some substances which had not been so studied previously. The effect of these choleretic and hydrocholeretic substances on the bromsulphthalein disappearance graph in dogs has been determined. It appears that a number of these act partly as competitive substances (in the doses given) for protein sites within the liver cell, and also act partly by effecting liver cell damage of a transient type.

The place of bromsulphthalein as a test of liver function has been reviewed and the use of the mathematical model as an aid in the quantitative assessment of liver damage or impairment has been discussed.

XIV. THE MATHEMATICAL APPENDIX

by

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Mathematical treatment of results. Suppose that at time t, x, y and z be the total amounts of BSP in the blood, in the liver and in the bile. It is assumed that under normal conditions :-

(i) all rates of transfer of BSP are proportional to the amounts of BSP from which the transfer occurs;

(ii) the amount of BSP transferred to extra-hepatic or extra-vascular tissue is negligible.

Let the constants of proportionality for the rates of transfer (i) from blood to liver, (ii) from liver back to blood, and (iii) from liver to bile be a, b and h respectively. Then following the completion of an injection of BSP into the blood, the above transfers are governed by the equations.

$$\left.\begin{aligned} \frac{dx}{dt} &= -ax + by \\ \frac{dy}{dt} &= -(b + h)y \\ \frac{dz}{dt} &= hy \end{aligned}\right\} \quad \ldots\ldots\ldots\ldots\ldots\ldots (1)$$

These equations have the solutions

$$x = \frac{(a - k_2)\,x_o - by_o}{k_1 - k_2}\, e - k_1 t + \frac{(k_1 - a)\,x_o + by_o}{k_1 - k_2}$$

$$y = \frac{(k_1 - a)\,y_o - ax_o}{k_1 - k_2}\, e - k_1 t + \frac{(a - k_2)\,y_o + ax_o}{k_1 - k_2} \quad \ldots\ldots(2)$$

$$z = x_o + y_o + z_o - y - y$$

The general solution of x in equation (2) is

$$x = Ae^{m_1 t} + Be^{m_2 t}$$

where m_1 and m_2 are roots of

$$k^2 + (a + b + h)k + ah = 0$$

but as the values m_1, m_2 are always negative in physiological conditions, it is more convenient to write

$$x = Ae^{-k_1 t} + Be^{-k_2 t}$$

where $k_1 = m_1$ and $k_2 + m_2$

and therefore k_1 and k_2 are roots of the equation

$$k^2 - (a + b + h)k + ah = 0 \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots(3)$$

It is found experimentally that k_1 is much greater than k_2 and so $e - k_1 t$ attenuates very much more quickly than $e - k_2 t$ and the last

term in the equation for x, in (2) above, is ultimately dominant. For large values of t, the first term may be neglected, so that

$$\ln x \doteq \ln \left(\frac{((k_1 - a)\, x_o + by_o)}{k_1 \quad k_2} \right) - k_2 t \quad \ldots\ldots\ldots\ldots\ldots\ldots (4)$$

Provided that the ratio

$$R = \frac{(a - k_2)\, x_o - by_o}{(k_1 - a)\, x_o + by_o}$$

is sufficiently large, the first term in x, (2 above) will be dominant initially, and thus for small values of t

$$\ln x \doteq \ln \left(\frac{((a - k_2)\, x_o - by_o)}{k_1 - k_2} \right) \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (5)$$

Equations (4) and (5) indicate that the graph of ln x shows an asymptotic approach to the line given by (4) for large values of t, an asymptotic approach to the line (5) for small values of t, and an intermediate part where the graph changes from one asymptote to the other. This gives a graphical method of determining k_1 and k_2 (see Figure 32); and, if x_o and y_o be known, a, b and h can be deduced.

As the ratio R decreases, however, the asymptote (5) is less and less likely to be evident and since R decreases as y_o increases, it follows in practice that if a second or third injection is given

before all the BSP from previous injections is excreted from the liver, the curve of ln x subsequent to later injections will show the values of k_1 less accurately. This explains why graphically (Figure 62) there appears to be a greater alteration of the initial rapid phase following the second and third injections than of the later slower phase, which is apparently unaltered. The constants of proportionality for the rates of transfer (i) from blood to liver, (ii) from liver to blood and (iii) from liver to bile, namely a, b and h respectively, are unaltered. Following the first injection the rate of the rapid phase is so high that there are seldom more than two points on the graph which contain large contributions from the first term in x (equation 2 above); after subsequent injections, these early points contain relatively larger contributions from the second term in x, so that the ratio R is not large. When the graphs obtained following the second and third injections are examined (Figure 62), it can be seen that the curves tend to identify the line joining the first few points on it as the asymptote given by equation 5 above, whereas in reality after second and later injections these points lie on the curves portions (BC) of the graph joining the two asymptotes (AB) and (CD).

In practice, an injection takes a few seconds to administer and before it is completed some BSP will have found its way out of

the blood. Strictly speaking, therefore, y_o and z_o as defined above are not zero but if for the present purpose it be assumed that they are, then x_o is equal to the total amount of BSP injected. This is equivalent to assuming that the injection is made instantaneously. With this limitation, then

$$x = x_o \left(\frac{(a - k_2}{(k_1 - k_2} e - k_1 t + \frac{k_1 - a}{k_1 - k_2} e - k_2 t \right) \Big) \Big)$$

$$y = \frac{ax_o}{k_1 - k_2} (e - k_2 t - e - k_1 t) \Big) \ldots\ldots (6)$$

$$z = x_o - x - y$$

In both (2) and (6), the third equation involves the assumption that all the BSP is to be found in the blood, liver or bile, the extra-hepatic or extra-vascular uptake being regarded as negligible.

In the preceding part of the mathematical treatment, the total amounts of BSP in the blood, liver and bile have been considered. Experimentally, the concentrations of BSP at various times (t) are obtained. From these plasma concentrations, it is possible to derive the plasma content of dye as explained in the following paragraph.

Assuming the plasma volume remains constant throughout the experiments, the observed plasma concentrations of BSP (C_x) say,

are directly proportional to the total amount of x in the circulation. Let the factor of proportionality be λ, so that

$$C_x = \lambda x \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots(7)$$

From the first equation of (6) is obtained

$$C_x = Ae^{-k_1 t} + Be^{-k_2 t} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots(8)$$

where ~~$Ae^{-k_1 t}$~~ ~~$Be^{-k_2 t}$~~

$$A = \frac{\lambda x_0 (a - k)}{k_1 - k_2} \quad \text{and} \quad B = \frac{\lambda x_0 (k_1 - a)}{k_1 - k_2} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots(9)$$

The asymptotic behaviour of the curves of ln C_x plotted against t is, of course, similar to those of ln x already described (in the paragraph following (5)).

The constants (A, B, k_1, k_2, a, b, h and λ) can now all be obtained by the following procedure :-

(i) To the observed curves of C_x, estimated values of A, B, k_1 and k_2 are fitted.

(ii) Since $\frac{A}{B} = \frac{a - k}{k_1 - a}$

$$\therefore a = \frac{Ak_1 + Bk_2}{A + B}$$

whence a is determined.

(iii) The relations $k_1 k_2 = ah$ and $k_1 + k_2 = a + b + h$, which follow from (3) next yield h and b.

(iv) Finally, since from (9)

$A + B = \lambda x_o$

and x_o (the dose of BSP) is known, λ can be determined.

Thereafter the experimentally-found concentrations (C_x) can be converted into estimates of actual quantities (x); finally, y and x (liver and bile content of BSP respectively) can be calculated from the second and third equations of (6).

Unfortunately, the numerical problem of determining the parameters k_1, k_2, a, b and h with mathematical precision is a difficult one. The results given in this paper have been obtained by using the values of A, B, k_1 and k_2 determined graphically as the starting point of a method of finding the values which gave the best fit of the observed data using the method of least squares. However, the usual normal equations are non-linear and the solutions have been found by making many trial solutions with values near those given graphically. This is a tedious 'searching' technique, and use has been made of an electronic computer because the desk calculation of the results of each experiment was found to take a skilled numerical analyst over a day.

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